RETINOID-INDUCED APOPTOSIS AND PROLIFERATION OF HEPATOCYTES ARE MEDIATED BY DISTINCT NUCLEAR RECEPTORS

BY

Pengli Bu

Submitted to the Graduate Degree Program in Pharmacology, Toxicology, and Therapeutics and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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LIST OF ABBREVIATIONS

ADFP	adipose differentiation-related protein
ADH	alcohol dehydrogenase
AF	activation function
Akt inhibitor	1,3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one trifluoroacetate salt hydrate
APC	adenoma polyposis colitis
APL	acute promyelocytic leukemia
BrdU	5-bromo-2'-deoxyuridine
ChIP	chromatin immunoprecipitation
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
CYP	cytochrome P450
DBD	DNA binding domain
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	dimethyl sulfoxide
DR	direct repeat
EGF	epithelial growth factor
ERK	extracellular signal-regulated kinase
FABP	fatty acid binding protein

FBS	fetal calf serum
FXR	farnesoid X receptor
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HCC	hepatocellular carcinoma
HDAC	histone deacetylase
HGF	hepatocyte growth factor
HRE	hormone response element
LBD	ligand binding domain
LXR	liver X receptor
MAPK	mitogen-activate protein kinase
MEM	Minimum Essential Medium
MMP	mitochondrial membrane permeabilization
NBRE	NGFI-B response element
NCoR	nuclear receptor corepressor
NES	nuclear export signal
NGFI-B	nerve growth factor-induced clone B
NLS	nuclear localization signal
NurRE	Nur-responsive element
PARP	Poly ADP-ribose polymerase
PCN	pregnenolone 16α-carbonitrile
PDK-1	phosphoinositide-dependent protein kinase 1
PH	partial hepatectomy

PI3K	phosphoinositide 3-kinase
PML	promyelocyte leukemia protein
PPAR	peroxisome proliferator-activated receptor
PXR	pregnane X receptor
RA	retinoic acid
RALDH	retinaldehyde dehydrogenase
RAR	retinoic acid receptor
RARE	retinoic acid response element
RXR	retinoid X receptor
SMRT	silencing mediator of retinoid and thyroid receptors
STAT	signal transducer and activator of transcription
TCPOBOP	1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene
TGF	transforming growth factor
TNF	tumor necrosis factor
TR	thyroid hormone receptor
TTNPB	4-(E-2-[5,6,7,8-tet-rahydro-5,5,8,8-tetramethyl-2-naphthalenyl]-1-propenyl) benzoic acid
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	vascular endothelial growth factor
VDR	vitamin D receptor

ABSTRACT

Retinoids, derivatives of vitamin A, are important signaling molecules involved in the regulation of cellular homeostasis including differentiation, apoptosis, and proliferation. The first part of this dissertation is focused on the mechanism underlying the differential susceptibilities of human hepatocellular carcinoma (HCC) cells to the apoptotic effect of a synthetic retinoid, fenretinide. The second part of this dissertation aims to investigate the mechanism responsible for 13-cis retinoic acid-induced hepatocyte proliferation.

Retinoids are used clinically to treat several types of cancer; however, their effects on liver cancer have not been fully characterized. To investigate the therapeutic potential of retinoids on HCC, the apoptotic effect of a panel of retinoids on three human HCC cell lines was evaluated and underlying mechanisms were investigated. Our results reveal that fenretinide effectively induced apoptosis in Huh-7 and Hep3B cells but not in HepG2 cells. Gene expression analysis of a panel of nuclear receptors demonstrates that the basal and inducibility of retinoic acid receptor (RAR) β expression positively correlate with the susceptibilities of HCC cells to fenretinide, thus suggesting a role of RARβ in this molecular determining process. Furthermore, transactivation of the RXRα/RARβ-mediated pathway in the reporter gene assay establishes fenretinide as an effective ligand. Fenretinide also increases the binding of RARβ to its cognate DNA response element (RARE, DR5) as demonstrated by chromatin immunoprecipitation (ChIP) assay. Knockdown of the endogenous RARβ expression with siRNA markedly impairs fenretinide-induced

apoptosis in the sensitive Huh-7 cells. Therefore, these results demonstrate that fenretinide activates RARβ and induces RARβ-dependent apoptosis in sensitive Huh-7 cells. These findings suggest a novel role for RARβ as a tumor suppressor by mediating the signals of certain chemotherapeutic agents.

Besides RARβ, one orphan nuclear receptor, Nur77, also seems to contribute to the differential sensitivities of human HCC cells to fenretinide-induced apoptosis. Our results clearly show that the expression of Nur77 positively correlates with the sensitivities of HCC cells to fenretinide. Moreover, the subcellular distribution patterns of Nur77 between Huh-7 and HepG2 cells in response to fenretinide were characterized and compared. It appears that Nur77 mediates the apoptotic effect of fenretinide in sensitive Huh-7 cells via actively targeting mitochondria whereas it confers HepG2 cells resistance by specifically accumulating in the nucleus. In addition, Nur77 knockdown by siRNA in Huh-7 cells demonstrates that the proapoptotic function of Nur77 was required for the full execution of fenretinide-induced apoptosis. Therefore, Nur77 exerts opposing effects in HCC cells in response to fenretinide, which is, at least in part, responsible for the observed differential susceptibilities of HCC cells.

Although retinoids cause growth arrest and differentiation in various cancer cells, they are also known to cause hepatomegaly in humans and rodents. Thus, the mechanism by which retinoids induce hepatocyte proliferation was examined. 13-cis retinoic acid (13-cis RA) stimulates proliferation of Hep3B cells, concomitant with elevation in DNA synthesis and cell cycle progression. *In vivo*, 13-cis RA increases

hepatic DNA synthesis after 5- and 10-day treatments. The high expression ratio of fatty acid binding protein 5 (FABP5) to cellular retinoic acid binding protein II (CRABP II) in Hep3B cells and in mouse livers prior to 13-cis RA treatment suggests preferred activation of peroxisome proliferator activated receptor β (PPARβ) over retinoic acid receptor (RAR) by 13-cis RA. The ratio of FABP5/CRABP II is further increased after 13-cis RA treatment. Moreover, 13-cis RA activates PPARβ and induces the expression of PPARβ target genes including phosphoinositide-dependent protein kinase 1 (PDK-1) in Hep3B cells and mouse livers. Following induction of PDK-1, Akt is phosphorylated and activated in both Hep3B cells and mouse livers. Over-expression of PPARβ in Hep3B cells further enhances 13-cis RA-induced DNA synthesis. Akt inhibitor blocks 13-cis RA-induced Akt activation and proliferation in a dose-dependent manner, but does not interfere with induction of PPARβ target genes indicating PPARβ is upstream of Akt. Activation of PPARβ/PDK-1/Akt cascade is thus responsible for the proliferative effect of 13-cis RA. Therefore, 13-cis RA promotes hepatocyte proliferation through activation of PPARβ.

CHAPTER ONE

GENERAL INTRODUCTION

OUTLINE

GENERAL INTRODUCTION

1.1 Retinoids

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1.1 Retinoids

1.1.1 The terminology of retinoid

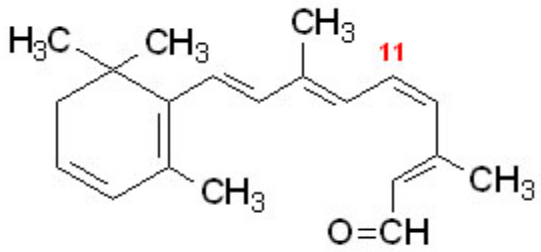
Retinoids, derivatives of vitamin A, are generally recognized as nutrients essential for life. The term "retinoids" introduced by Sporn et al. in 1976 was designated by the International Union of Pure and Applied Chemistry - International Union of Biochemistry (IUPAC-IUB) to include "compounds consisting of four isoprenoid units joined in a head-to-tail manner; all retinoids may be formally derived from a monocyclic parent compound containing five carbon-carbon double bonds and a functional terminal group at the terminus of the acyclic portion." However, several synthetic compounds, which do not fit into this definition of retinoids, are much more active than retinol or retinoic acid in several assays for vitamin A or retinoid activity. Sporn and Roberts (1985) thus proposed that: "a retinoid should be defined as a substance that can elicit specific biologic responses by binding to and activating a specific receptor or set of receptors". Today most researchers use a combination of the above two definitions, that is, the class of retinoids consists of retinol analogs (with or without biologic activity) but also of several compounds that are not closely related to retinol but elicit biological vitamin A or retinoid activity (Blomhoff and Blomhoff, 2006).

1.1.2 Retinoid structure, physiological functions and clinical relevance

The function of vitamin A in the visual process was resolved several decades ago. 11-cis retinal or closely related retinoid such as 11-cis-3-hydroxy-retinal serves as the chromophore of various visual pigment complexes which also contain a molecule called opsin in the eye. When a photon of light hits the complex, the retinal changes its conformation from 11-cis to all-trans, thus initiating a chain of events that results in the transmission of an impulse up the optic nerve (see Figure 1.1 for the structures of some natural retinoids).

Besides their well-known phototransduction function, retinoids have important functions in various biological processes including reproduction, embryonic growth and development, maintenance of epithelial homeostasis, and immune competence (Blomhoff and Blomhoff, 2006; Marill et al., 2003; Zile, 1998). Early experimental and clinical evidences showed that vitamin A-deficiency and vitamin A-excess dramatically change the differentiation of epithelial cells and result in congenital malformations. Remarkably, a link of the physiological function of vitamin A to the development of heart, embryonal circulatory, central nervous system, and the regulation of heart asymmetry has been unequivocally established with the use of the vitamin A–deficient avian embryo model (Zile, 1998). In humans, when intake of vitamin A is inadequate to meet the body's needs, clinical signs of vitamin A deficiency are manifested as several ocular features (xerophthalmia) and a generalized impaired resistance to infection (Blomhoff and Blomhoff, 2006). Both symptoms indicate a role for retinoid function in visual and immune systems.

(A)



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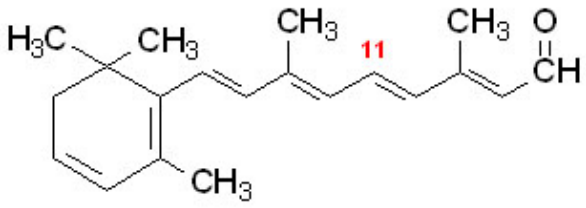

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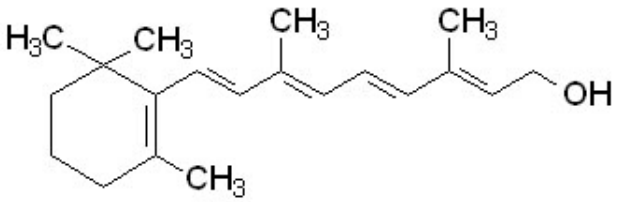

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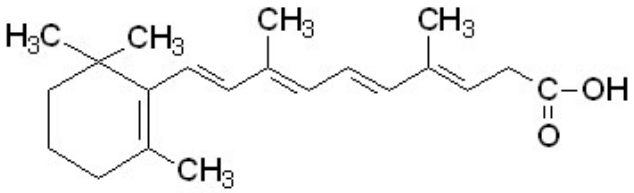


Figure 1.1 Structure of some natural retinoids: (A) 11-cis retinal, (B) all-trans retinal, (C) retinol (vitamin A), and (D) all-trans retinoic acid. Adapted from (Blomhoff and Blomhoff, 2006).

Hypervitaminosis A can also occur when too much retinoids are in the body. It has been shown that intake marginally above the recommended dietary intake is associated with embryonic malformations, reduced bone mineral density, and increased risk for hip fracture (Blomhoff and Blomhoff, 2006). The classical signs of hypervitaminosis A occur in skin, nervous system, musculo-skeletal system, circulation, and internal organs, as well as in the fetus following an excessive dietary intake or an intake of drugs containing large doses of specific retinoids (Blomhoff and Blomhoff, 2006).

Clinically, retinoids are effective in treating dermatological diseases such as acne and psoriasis and certain types of cancer including acute promyelocytic leukemia, cutaneous T cell lymphoma, and Kaposi's sarcoma (LeadDiscovery, 2003).

There are two types of retinoic acid used clinically. One is all-trans retinoic acid, also known as tretinoin and its brand names include Retin A, Retin A Micro, Tretinoin Gel, Renova, and Retiva. The other one is 13-cis retinoic acid, also known as isotretinoin, and its brand names include Accutane, Roaccutane, Accure, and IsotrexGel. Both drugs are the acid form of vitamin A (retinol) and are used to treat skin disorders. Topical tretinoin is used to treat mild to moderate acne and skin that has been damaged by excessive sun exposure. Isotretinoin is used to treat severe acne that is resistant or minimally responsive to the conserved treatments such as creams, topical or oral antibiotics (LeadDiscovery, 2003).

1.1.3 Retinoid metabolism

No animal species has the capability for *de novo* vitamin A synthesis. However, plants and microorganisms such as some bacteria and fungi can synthesize carotenoids, which can be converted to retinoids in animals. The carotenoids represent a large group of pigments that are responsible for the yellow, orange, red, or purple colors of many vegetables, fruits, and flowers (Fraser and Bramley, 2004). Many of these carotenoids are absorbed and stored in animals, and often to such a degree that they give color to animal tissues. For example, lycopene is concentrated in human prostate, β-carotene in chicken egg yolk, and astaxanthin and canthaxanthin in salmon flesh and flamingo feathers (Fraser and Bramley, 2004). Animals obtain vitamin A by eating plant and animal tissues which contain carotenoids and/or retinoids converted from carotenoids. Especially, retinoids, such as retinyl esters and retinol that are rich in fish, avian, and mammalian livers, also contribute to the dietary intake of vitamin A.

There are over 600 carotenoids in nature and approximately 50 of these can be metabolized to vitamin A. β-carotene is the most prevalent carotenoid in the food supply that has pro-vitamin A activity. Moore first described that β-carotene can be converted to retinoids in the small intestine (Moore, 1930). Later, a central cleavage mechanism responsible for yielding two molecules of retinal from one molecule of β-carotene was characterized by two independent groups (Goodman and Huang, 1965; Olson and Hayaishi, 1965). Thus retinal, formed in the intestine as a product of carotenoid cleavage, is subsequently reduced to retinol and taken up by enterocytes.

Dietary retinyl esters are hydrolyzed to retinol in the intestinal lumen prior to uptake by the enterocytes (Blomhoff and Blomhoff, 2006).

In enterocytes, most retinol is re-esterified with long chain fatty acids (mainly palmitate) by the enzyme lecithin:retinol acyl transferase (LRAT). Cellular retinol binding protein type II (CRBP II, a protein primarily expressed in the intestine) facilitates this process by binding to retinol and directing it to LRAT (Herr and Ong, 1992). A majority of the retinyl esters are then incorporated into chylomicrons and secreted into general circulation (Blomhoff et al., 1982). Plasma chylomicron remnants that contain most of retinyl esters from diet are taken up by hepatocytes (Blomhoff et al., 1982) and to a less extent by bone marrow, peripheral blood cells, spleen, adipose tissue, skeletal muscle and kidney (Paik et al., 2004).

In mammals, 50-80% of the body's total retinoids are stored in hepatic stellate cells as retinyl ester (Blomhoff and Blomhoff, 2006; Fontana and Rishi, 2002). In hepatocytes, retinyl esters are hydrolyzed and associated with retinol binding protein (RBP) followed by secretion into plasma. A large portion of hepatic retinol is re-esterified in to retinyl ester and packed together in cytoplasmic lipid droplets in stellate cells (Blomhoff and Blomhoff, 2006).

It is generally believed that active retinoid metabolites are synthesized in target cells. All-trans retinoic acid is the major active cellular retinoid metabolite synthesized from retinol by a two-step reaction. The first also the rate-limiting step in this process is the oxidation of retinol to retinal. Several alcohol dehydrogenases (ADH1, 3, and 4) are able to catalyze this reaction. The second step is the oxidation

of retinal to retinoic acid by retinaldehyde dehydrogenases (RALDH) such as RALDH 1, 2, 3, and 4 depending on tissues and cell types (Niederreither et al., 2002).

Catabolism of retinoic acid controls the level of this signaling molecule in cells and tissues. Cytochrome P450 enzyme (CYP) 26A1 was shown to metabolize retinoic acid into polar metabolites including 4-hydroxy retinoic acid, 4-oxo retinoic acid, 18-hydroxy retinoic acid, 5,6-epoxy retinoic acid, and 5,8-epoxy retinoic acid (Swindell and Eichele, 1999). The promoter region of CYP26A1 contains two functional retinoic acid response elements (RARE) and thus the transcription of this enzyme is inducible by retinoic acid. Therefore, it seems that CYP26A1 senses the cellular level of retinoic acid and regulates its degradation accordingly. Other cytochrome P450 enzymes (CYP26B1 and 26C1) that are able to metabolize retinoic acid were subsequently identified (Taimi et al., 2004; White et al., 2000). The expression patterns of CYP26A1, CYP26B1, and CYP26C1 do generally not overlap, thus suggesting different roles for each of the CYP26 enzymes in the catabolism of retinoic acid (Reijntjes et al., 2004). Interestingly, cellular retinoic acid binding protein type I (CRABP-I) seems to facilitate retinoic acid degradation because overexpression of the CRABP-I protein in transfected F9 stem cell lines resulted in a higher level of degradation of retinoic acid (Boylan and Gudas, 1991; Boylan and Gudas, 1992).

1.1.4 Fenretinide

Fenretinide (N-(4-hydroxyphenyl) retinamide, also known as 4HPR) is a synthetic analog of all-trans retinoic acid that was first produced by R. W. Johnson Pharmaceuticals in the late 1960's (see Figure 1.2 for structure comparison between all-trans retinoic acid and fenretinide). The substitution of an amide-linked 4-hydroxyphenyl group for the carboxyl group of all-trans retinoic acid markedly reduces adverse effects such as liver toxicity. Furthermore, fenretinide lacks the ability to induce point mutations or chromosomal aberrations and thus is not believed to be genotoxic. These qualities suggest that fenretinide could be useful for long-term chemoprevention (Hail et al., 2006). Indeed, in animal models, fenretinide has demonstrated chemopreventive effects against carcinogenesis of breast, prostate, pancreas, and skin (Abou-Issa et al., 1995; McCormick et al., 1987; McCormick and Moon, 1986; Ohshima et al., 1985). Moreover, in clinical trials, fenretinide slowed the progression of prostate cancer in men, protected against the development of ovarian cancer and a second breast malignancy in premenopausal women, and prevented relapse and the formation of secondary primary lesions in patients following the surgical removal of oral leukoplakia (Chiesa et al., 2005; Chodak et al., 1993; Veronesi et al., 1999).

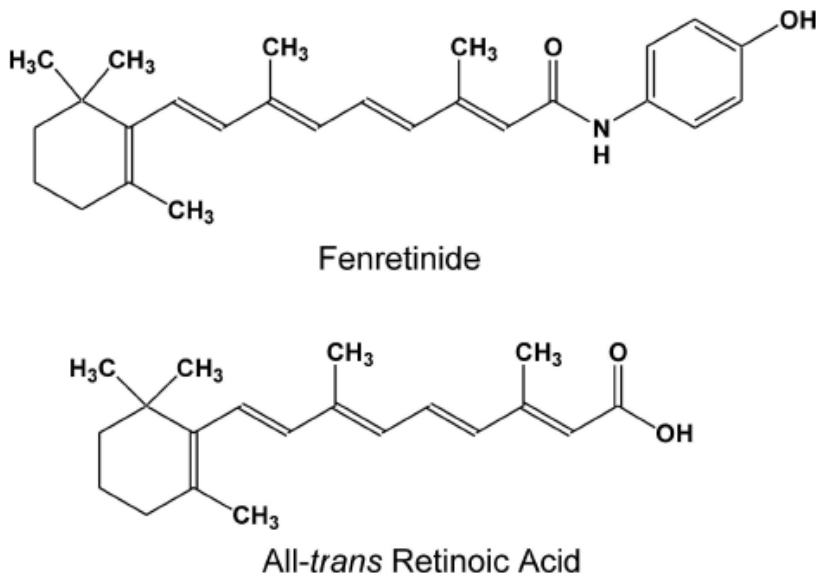


Figure 1.2 Structure comparison between all-trans retinoic acid and fenretinide.

Unlike natural retinoid such as all-trans retinoic acid that induces differentiation and/or growth arrest of target cells, fenretinide causes apoptosis in various cancer cells. The *in vitro* apoptotic activity of fenretinide appears to be selective in transformed/malignant cells while sparing their normal counterparts. This selective effect has been observed in normal fibroblasts, lymphocytes, hepatocytes, and cervical epithelial cells (Hail et al., 2006). Thus, the excellent tolerance and the targeted apoptotic activity of fenretinide may confer it as potentially valuable in both chemoprevention and cancer chemotherapy.

1.2 Nuclear receptors

1.2.1 Organization and nomenclature of the nuclear receptor superfamily

Multicellular organisms require specific genetic programs to properly organize the complex body plan during embryogenesis and maintain its properties and functions during the entire life span. Unlike signaling molecules such as growth factors, neurotransmitters, and peptide hormones, which bind to membrane receptors and induce the activity of intracellular kinase cascade; various small, hydrophobic signaling molecules such as steroid hormones, certain vitamins, and metabolic intermediates that diffuse into or are generated within the target cells, bind to cognate members of a large family known as nuclear receptors superfamily (Germain et al., 2003; Germain et al., 2006c).

By 2006, 48 nuclear receptors have been identified in human genome (Figure. 1.3) (Germain et al., 2006c). They constitute a family of ligand-dependent transcription factors that share a modular structure of five to six conserved domains with specific functions (Evans, 2005; Germain et al., 2006c; Mangelsdorf et al., 1995). Their most prominent distinction from other transcription factors is that they can specifically bind small hydrophobic molecules. Their ligands serve as regulatory signals that change the transcriptional activities of the corresponding nuclear receptors upon binding. For some time, a distinction was made between classic nuclear receptors with known ligands and so-called "orphan" receptors, referring to the nuclear receptors without or with unknown ligands. However, in recent years,

ligands for many of these orphan receptors have been identified, thus making this distinction rather superficial. Moreover, the classification of nuclear receptors into six to seven phylogenetic subfamilies with groups that comprise both orphan and non-orphan receptors further dismisses such discrimination (Cell, 1999; Germain et al., 2006c). Therefore, the classification of nuclear receptors is done by virtue of the homology to other family members, with the DNA- and ligand-binding domains (DBDs and LBDs) having the highest evolutionary conservation (Germain et al., 2003; Germain et al., 2006c).

Interestingly, some recently identified ligands for some "orphan" nuclear receptors turned out to be metabolic intermediates. It appears that in certain systems the control of build-up, break-down and storage of metabolic active substances is regulated at the level of gene transcription. Prominent metabolic ligands are bile acids, fatty acids, eicosanoides, and oxysterols. The group of steroid hormones includes estrogens, progestins, mineralocorticoids, glucocorticoids, androgens, and ecdysterones. Examples for vitamin–derived ligands include vitamin D3 (for vitamin D receptor) and the vitamin A derivative, retinoic acid (for retinoid receptors such as retinoic acid receptor and retinoid X receptor). Therefore, nuclear receptors function in signaling pathways covering endocrine (steroid hormone receptors), autocrine or paracrine (retinoid receptors), and intracrine (metabolic receptors such as liver X receptor (LXR), farnesoid X receptor (FXR), pregnane X receptor (PXR), and proxisome proliferator-activated receptor (PPARs)) (Germain et al., 2003; Germain et al., 2006c).

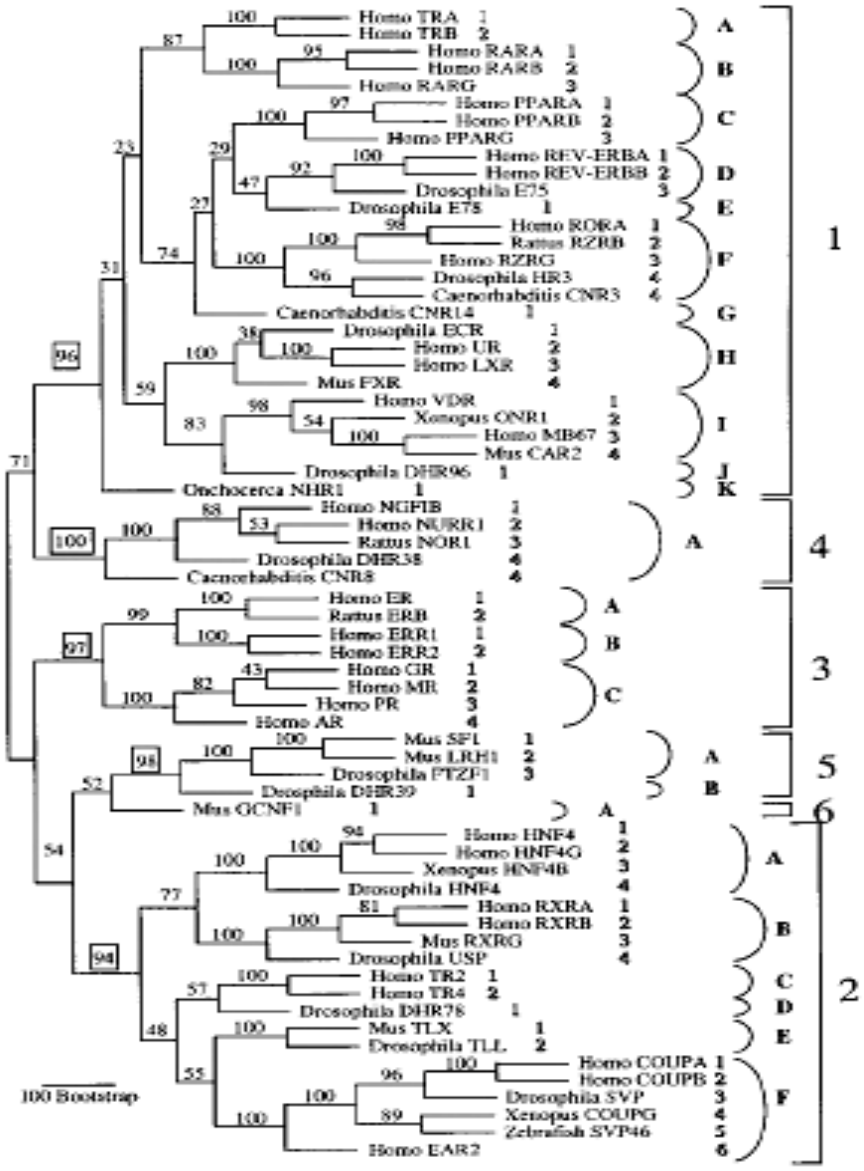


Figure 1.3. Phylogenetic tree of 65 nuclear receptor genes in vertebrates, arthropoids and nematodes. For a detailed description, see Nuclear Receptors Nomenclature Committee (Cell, 1999) and the regular updates at http://www.ens-lyon.fr/LBMC/LAUDET/nomenc.html. Adapted from (Germain et al., 2003).

1.2.2 Modular structure and functions of nuclear receptors

As schematically depicted in Figure 1.4, nuclear receptors are generally composed of six highly conserved regions (A to F).

The N-terminal A/B region harbors one (or more) autonomous transcriptional activation function (AF-1), which can activate transcription in a constitutive manner when linked to a heterologous DNA-binding domain. However, in the context of the full-length receptor, AF-1 is silent in the absence of agonists and certain antagonists. When comparing nuclear receptors from different subfamilies and groups, the A/B region displays the weakest evolutionary conservation, and the distinction between A and B regions is not always clear. A/B regions differ significantly in their length, ranging from 23 (vitamin D receptor,) to 550 (androgen receptors, mineralocorticoid receptors, and glucocorticoid receptors) amino acids. A/B regions are subject to differential promoter usage and alternative splicing, and the majority of known NR isoforms differ in their N-terminal region. Moreover, the N-terminus of nuclear receptors has been found as subject of posttranslational events such as phosphorylation. Finally, the activation function AF-1 displays cell type-specific action (Germain et al., 2003; Germain et al., 2006c).

The highly conserved C domain harbors the DNA binding domain (DBD) of nuclear receptors, which confers sequence-specific DNA recognition. This domain has been extensively studied, especially with respect to its recognition of selective response elements and its dimerization properties. Several X-ray and NMR data sets are available for different nuclear receptor C domains in their DNA complexed and

uncomplexed forms (Germain et al., 2003; Germain et al., 2006c). The DBD is generally composed of two zinc-finger motifs, the N-terminal motif Cys-X2-Cys-X13-Cys-X2-Cys (CI) and the C-terminal motif Cys-X5-Cys-X9-Cys-X2-Cys (CII); in each motif, two cysteine residues chelate one Zn^{2+} ion. Within the C domain, several sequence elements (termed P-, D-, T- and A-boxes) have been identified that define or contribute to (1) response element specificity, (2) a dimerization interface within the DBD, and (3) contacts with the DNA backbone and residues flanking the DNA core recognition sequence (Germain et al., 2003; Germain et al., 2006c).

The D region of nuclear receptors is less conserved than the surrounding regions C and E. This domain appears to represent a "hinge" between the highly structured C and E domains. It might allow the DBD and ligand binding domain (LBD) to adopt different conformations without creating spatial hindrance problems. Region D of certain nuclear receptors contains a nuclear localization signal (NLS), or at least some elements of a functional NLS. The intracellular localization of nuclear receptors is a result of a dynamic equilibrium between nuclear-cytosol and cytosol-nuclear shuttling (Guiochon-Mantel et al., 1994). At equilibrium, the large majority of nuclear receptors reside in nucleus, while some steroid receptors (androgen, glucocorticoid, and mineralocorticoid receptor) and the so-called orphan nuclear receptor CAR apparently reside within cytosol in the absence of their cognate ligands and translocate to the nucleus in a ligand-induced manner (Germain et al., 2003).

The hallmark of a nuclear receptor is its LBD in the E region. This domain is highly structured and encodes a number of distinct functions, most of which operate

in a ligand-dependent manner. The LBD harbors the ligand-dependent activation function AF-2, a major dimerization interface and often a repression function (Laudet and Gronemeyer, 2001). Detailed molecular insights into the structure-function relation of nuclear receptors have been gained by the elucidation of the crystal structures of the E region alone or in the presence of agonists, antagonists and coregulator peptides (Germain et al., 2002).

The role of the C-terminal region F is usually unknown. Not all receptors possess a C-terminal region F, which displays little evolutionary conservation. The length of this region is variable depending on specific nuclear receptors. In addition, there are no clues as to the function of the C-terminal sequence. Recent studies suggest that the F region might play a role in coactivator recruitment to the E domain and in determining the specificity of the LBD coactivator interface (Peters and Khan, 1999; Sladek et al., 1999). It seems clear that this domain also inherits little structural features. It is tempting to speculate that it may somehow fine-tune the molecular events associated with the transcriptional properties of the E domain, or the entire receptor (Nichols et al., 1998).

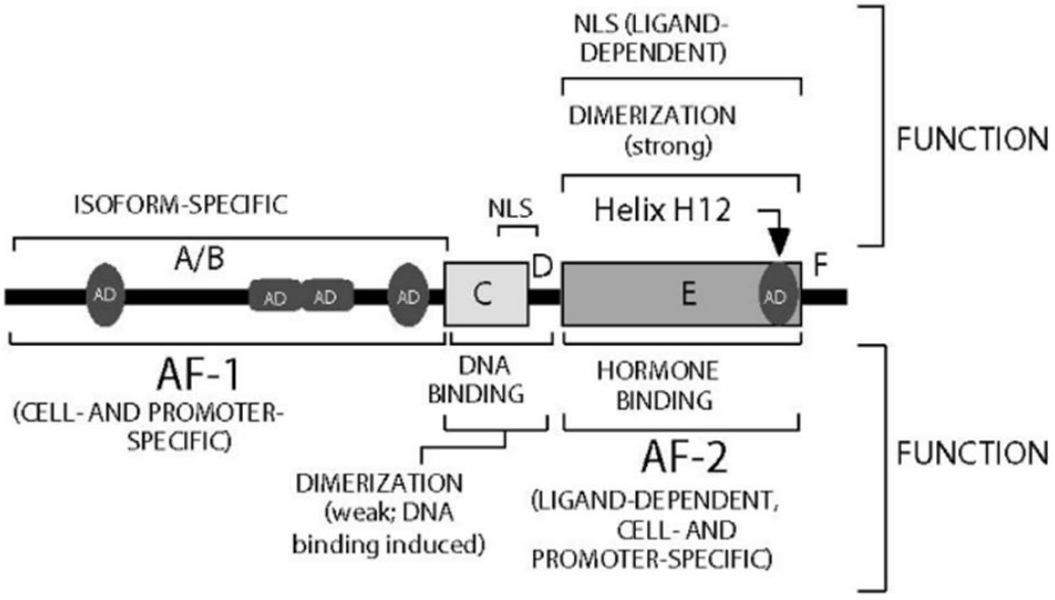


Figure 1.4 Schematic illustration of the structural and functional organization of nuclear receptors. Adapted from (Germain et al., 2003).

1.2.3 Nuclear retinoid receptors

Retinoids have emerged as important signaling molecules in the regulation of cellular homeostasis and development. During the past two decades, the knowledge on the mechanism of retinoid action has been greatly expanded due to the discovery and characterization of nuclear retinoid receptors and the consensus RAREs in retinoid target genes (Bastien and Rochette-Egly, 2004; Evans, 2005; Fontana and Rishi, 2002; Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995). The retinoid signaling is generally transduced by two members of the nuclear receptor superfamily, the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), working as RXR/RAR heterodimer. Both nuclear receptors consist of three subtypes (α, β, and γ). Interestingly, the sequence for each RAR subtype differs significantly from one another as they originate from different genes, but the sequences for each subtype are highly conserved between humans and mice. This indicates that these RAR subtypes may have distinct functions. Furthermore, for each subtype of RAR and RXR, several isoforms have been reported that are generated by differential promoter usage and/or alternative splicing and differ from one another in their N-terminal A/B region (Bastien and Rochette-Egly, 2004; Germain et al., 2006a; Germain et al., 2006b). RARs are activated by all-trans retinoic acid and its isomer 9-cis retinoic acid, while RXRs are only activated by 9-cis RA (Bastien and Rochette-Egly, 2004; Mangelsdorf and Evans, 1995; Marill et al., 2003). Like most nuclear hormone receptors, retinoid receptors exhibit a modular structure composed of six conserved regions designated A–F (Figure 1.4).

Almost all nuclear receptors primarily act through direct association with specific DNA sequences known as hormone response elements (HREs) (Evans, 1988). For the nonsteroid nuclear receptors, the HREs consist of a hexamer core consensus sequence AGGTCA that can be configured into a modular response motif. It was established that the most potent HREs for the nonsteroid nuclear receptors are direct repeats (DRs) of the core AGGTCA half-site (Evans, 2005). In the direct repeat paradigm, the spacing between the two half-site, not sequence difference, is the key ingredient to distinguishing the specific response elements for nuclear receptor dimers; which is referred to as the 1-5 rule (Figure 1.5). A compilation of observations from several laboratories demonstrate that the HREs for the vitamin D receptor (VDR), thyroid hormone receptor (TR), and retinoic acid receptor (RAR) are composed of DRs spaced by 3, 4, or 5 nucleotides (i.e., DR3, DR4, and DR5, respectively) (Umesono et al., 1991). Later on, it was observed that a DR1 serves as an RXR and peroxisome proliferator-activated receptor (PPAR) response element (PPRE) and a DR2 is a second retinoic acid response element (RARE) (Evans, 2005; Mangelsdorf and Evans, 1995).

Figure 1.5 Nonsteroid nuclear receptor heterodimers that fit into the direct repeat (DR) 1-5 rule. Adapted from (Evans, 2005).

In the absence of ligand, retinoid receptors are found primarily in the nucleus. They bind as asymmetric, oriented RXR/RAR heterodimer to retinoic acid response elements (RARE) composed typically of two direct repeats of a core hexamer motif, AGGTCA (Mangelsdorf and Evans, 1995). The classical RARE is a 5-nucleotide-spaced direct repeat (referred to as DR5) (Yang et al., 1991). However, RXR/RAR heterodimer also binds to direct repeats separated by 2 nucleotides (DR2). RXRs also bind to DR1 as RXR/RXR homodimers (Evans, 2005; Mangelsdorf and Evans, 1995). RAREs have been identified in the promoters of a large number of retinoid-target genes implicated in a wide variety of functions. The classical DR5 elements are found in the promoter of the RARβ gene itself, cytochrome P450 enzyme CYP26, and other genes (Bastien and Rochette-Egly, 2004). DR2 elements were identified in the CRBPI and CRABPII promoters. The only natural DR1 element binding RXR homodimer has been found in the rat CRBPII promoter (Mangelsdorf et al., 1991). On DR2 and DR5 elements (Figure 4B), RXR occupies the 5' hexameric motif, whereas the RAR partner occupies the 3' motif (Chambon, 1996; Laudet and Gronemeyer, 2001).

A model has been proposed to explain how retinoid receptors regulate gene expression (Dilworth and Chambon, 2001). In the absence of the ligand retinoid receptors bind DNA and repress transcription through the recruitment of the corepressors such as NCoR (nuclear receptor corepressors) and SMRT (silencing mediator of retinoid and thyroid receptors). Furthermore, the corepressors reside in or recruit high molecular weight complexes containing histone deacetylase (HDACs)

activities that increase the interaction of the N-terminal histone tails with the DNA and thus condense local chromatin structure. Therefore, to activate gene transcription, retinoid receptors will have to contend with the repressive chromatin structure in order to recruit transcription machinery. In this regard, the ligand-induced conformational changes in the receptors will cause the dissociation of corepressors and the coordinated and/or combinatorial recruitment of coactivators. The recruited coactivators will further interact with larger protein complexes containing chromatin modifying and remodeling activities to decompact the repressive chromatin structure and facilitate the positioning of the transcriptional machinery at the promoter (Bastien and Rochette-Egly, 2004; Dilworth and Chambon, 2001). The ligand-induced conformational changes also favor the interactions between RAR and RXR and therefore increase their binding affinity for dimerization and for cognate DNA response element (Bastien and Rochette-Egly, 2004).

Genetic approaches including targeted disruption (knockout) via homologous recombination were used to generate mice lacking nuclear retinoid receptors. These nuclear retinoid receptor null mice have led to significant advances in our knowledge of the physiological functions of the corresponding receptors (Kastner et al., 1995). Previously, the physiological functions of retinoids were mainly inferred from studies on vitamin A-deficient animal and from the defects caused by administration of pharmacological doses of retinoids (Means and Gudas, 1995). Vitamin A-deficient studies showed that vitamin A (retinol) is required during pre- and postnatal development and in adult life. After birth, retinol is indispensable for survival, growth,

reproduction, vision, and the maintenance of numerous tissues (Chambon, 1994; Means and Gudas, 1995). Null mutations of RARα, RARβ, or RARγ, and several isoform-specific knockouts of RAR have been generated (Kastner et al., 1995). Surprisingly, mice lacking RARα1, RARβ, RARβ2, or RARγ2 appear normal. In contrast, most of the double mutants exhibit a much more severe phenotype with markedly reduced viability (Kastner et al., 1995). Furthermore, almost all of the malformations observed in the fetal vitamin A-deficient syndrome are recapitulated in the different RAR double mutants. Therefore, these findings demonstrate that retinoic acid, the physiological ligand for RAR, is the active vitamin A metabolite during development and that the effects of vitamin A are mediated by RARs (Kastner et al., 1995).

Examination of RXRα null mice and various RXRα/RAR double mutants have revealed a convergence between RXR- and RAR-mediated signaling pathways because of the similarity in the phenotypes of the null mice. In addition, synergistic effects of RXRα and RAR double mutations strongly suggest that, at least in some instances, RXRα/RAR heterodimers act as functional units transducing the retinoid signaling *in vivo* (Kastner et al., 1995).

1.2.4 RXRα subordination

Many nuclear receptors use RXR as a heterodimer partner. In contrast to homodimerization, heterodimerization may allow, in principle, fine-tuning of NR actions by using combinatorial sets of ligands. However, whereas RAR agonists can autonomously activate transcription through RXR-RAR heterodimers, RXR is unable to respond to RXR-selective agonists in the absence of the RAR ligands. Consequently, RXR-selective ligands on their own could not trigger RXR/RAR heterodimer-mediated retinoid signaling events in various cell systems. Similarly, RXR cannot autonomously respond to its ligands in the RXR/TR and RXR/VDR heterodimers, unless the ligands of those heterodimer partners are present (Bastien and Rochette-Egly, 2004; Germain et al., 2003). This phenomenon, referred to as RXR subordination or RXR silencing, may be of great biological importance because it provides a way to avoid confusion among retinoic acid, thyroid hormone, and vitamin D3 signaling pathways. RXR subordination is, however, not due to an inability of RXR to bind its cognate ligand in DNA-bound heterodimers, as was originally proposed, because RXR ligand binding has been demonstrated to occur in such complexes. Studies have demonstrated that RXR can bind its ligand (holo RXR) and recruit coactivators in heterodimer with unliganded (apo) RAR. However, in the usual cellular environment, corepressors do not dissociate and thus compete with coactivators for binding. Corepressor binding to RAR prevents the holoRXR/apoRAR heterodimer from assembling a transcription-initiation complex (Germain et al., 2002). Consequently, the only way for RXR to activate transcription in response to its

ligands in RXR-RAR heterodimers is through synergy with RAR ligands (Germain et al., 2003). RXR subordination do not apply to all its heterodimer partners, as the ligand-induced RXR activity is permissive/sufficient in heterodimers with FXR, LXR, PPAR, or Nur77/NGFI-B (Germain et al., 2003; Germain et al., 2006c).

1.2.5 Orphan nuclear receptor Nur77

The Nur77 subfamily of the nuclear hormone receptor superfamily consists of three members NR4A1, NR4A2 and NR4A3. All of them belong to the so-called orphan nuclear receptors because no known ligands have been identified to date. NR4A1 is also known as Nur77, thyroid hormone receptor 3 (TR3), or nerve growth factor-induced clone B (NGFI-B); NR4A2 is also named as Nurr1, RNR-1, or TONOR; while NR4A3 has also been called Nor-1 or Minor (Hsu et al., 2004; Li et al., 2006; Maxwell and Muscat, 2006). The structure of these orphan nuclear receptors includes an activation domain AF-1 at their N-terminus region A/B, a DNA binding domain (DBD), and a ligand binding domain (LBD) at their C-terminus (Hsu et al., 2004). The amino acid sequences of NR4A1, 2, and 3 are highly conserved in the DBD domain (~91-95%), modestly conserved in the LBD domain (~60%), and very divergent in their activation function domain AF-1. All three members contain a functional nuclear localization signal (NLS) in their DBDs (Li et al., 2006). The LBD of Nur77 contains three nuclear export signals (3xNESs) indicating its potential to be actively shuttling between nucleus and cytosol. A Bcl-2 binding domain has also been identified in the LBD of Nur77, which suggests that this orphan nuclear receptor has the structural basis for direct interaction with the antiapoptotic protein Bcl-2 (Li et al., 2006). Nur77 binds to the consensus DNA sequence AAAGGTCA (NGFI-B response element, NBRE) as monomers, and to AAAT(G/A)(C/T)CA (Nur-responsive element, NurRE) as homodimer. Nur77 has also been shown to dimerize with RXR and bind to RARE (DR5) (Hsu et al., 2004). Nur77 family proteins are involved in a wide variety

of biological processes including cell survival and apoptosis. They are known immediate early genes induced by serum, growth factors and receptor engagement (Li et al., 2006; Maxwell and Muscat, 2006).

Remarkably, Nur77 has been demonstrated to play dual roles in controlling survival and death of cancer cells by a wealth of experimental data. It appears that the pro- or anti-apoptotic activity of Nur77 is determined by its subcellular localization. In the nucleus, Nur77 functions as an oncogenic survival factor by promoting cancer cell growth. In contrast, it becomes a potent killer when migrating to mitochondria, where it binds to Bcl-2 and converts the survival phenotype of Bcl-2 into proapoptotic, thus facilitating cytochrome c release and subsequent apoptosis induction (Lin et al., 2004). A retinoid derivative, 6-(3-(1-adamantyl)-4-hydroxyphenyl)-2-naphtha-lenecarboxylic acid (also known as AHPN/CD437), induces Nur77 migration from the nucleus to mitochondria and thus effectively triggers apoptosis induction in various cancer cells (Li et al., 2000). Furthermore, Nur77 translocation is highly controlled by its heterodimer partner RXR, suggesting a role of RXR ligands in regulating this process (Cao et al., 2004). Therefore, translocation of Nur77 from nucleus to mitochondria may illustrate a new mechanism in cancer cell apoptosis, and targeting Nur77 translocation may represent a potential strategy in cancer therapy (Moll et al., 2006; Zhang, 2007).

1.3 Apoptosis and its regulatory pathways

The term apoptosis is of Greek origin meaning "falling off or dropping off" of leaves from trees or petals from flowers. It was in 1972 that the term first appeared in the biomedical literature. A group of sharp-eyed scientists described a structurally-distinctive form of cell death that is responsible for cell loss within living tissues (Kerr et al., 1972). They noticed that these dying cells shared many common morphological features, such as cell shrinkage and chromatin condensation, transient but violent bubbling and blebbing of the cell surface, and separation of the cell into a cluster of membrane-bound bodies. Finally, the membrane-bound bodies, also known as apoptotic bodies, were rapidly engulfed by phagocytes under normal physiological condition. All of those features were distinct from the features observed in cells undergoing pathological necrotic cell death (see a more detailed comparison in Table 1.1). Thus they suggested that there might be a common and conserved endogenous cell death program that is responsible for these unique and conserved morphological features (Wyllie et al., 1980).

Table 1.1 Comparison of apoptosis with necrosis

Apoptosis	**Necrosis**
"Programmed"	"Accidental"
Physiological or pathological	Pathological
Cell shrinkage	Cell swelling
Condensation: membranes intact	Cytolysis: membranes destroyed
No inflammation	Inflammation
Energy required	Energy not required
Discrete DNA fragmentation	Randomized DNA

As knowledge accumulated and studies in cell death in the worms (C.elegans) and fruit flies (Drosophila) emerged, scientists came to agree that apoptosis is a regulated (programmed) form of cell death. As a counterbalance to cell proliferation,

apoptosis is necessary for multicellular organisms to get rid of excess cells to obtain successful organogenesis during embryonic development, to control cell number and tissue size, to maintain homeostasis at an adult stage, and to protect the organism from the threat of malignant cells during its whole life span. With apoptosis, all of the above can be achieved in a neat and prompt way with almost no harm to the organism (Hengartner, 2000). However, deregulation of apoptosis may result in pathological conditions. Excessive apoptosis may set the stage for diseases including acute and chronic degenerative diseases, immunodeficiency, and infertility; whereas too little apoptosis is associated with autoimmune disease and certain types of cancer.

There are two major molecular pathways controlling the initiation and execution of apoptotic cell death: the extrinsic or death-receptor mediated pathway and the intrinsic or mitochondria-mediated pathway (Figure 1.6). In hepatocytes, apoptosis is generally mediated through the mitochondria-mediated pathway (Green and Reed, 1998). The intrinsic pathway of apoptosis relies on mitochondrial membrane permeabilization (MMP) to release the apoptotic mitochondrial proteins including cytochrome c, endonuclease G, and second mitochondrial activator of caspases (Smac). It is worth mentioning that the Bcl-2 family members play a central role in the regulation of MMP and apoptosis. During cellular stress, antiapoptotic Bcl-2 family members (e.g., Bcl-2 and Bcl-XL) residing in the outer mitochondrial membrane can be functionally neutralized through a decrease in expression, or by the induction of proapoptotic Bcl-2 family members (e.g., Bax, Bad, and Bak). In this scenario, the ratio of proapoptotic family members to antiapoptotic family members

becomes greater allowing the formation of outer membrane channels by the proapoptotic Bcl-2 family members. Consequently, MMP is achieved and the apoptotic mitochondrial proteins get released into cytosol, which activate caspase-9 and the subsequent downstream caspase cascade to initiate apoptosis (Hail et al., 2006).

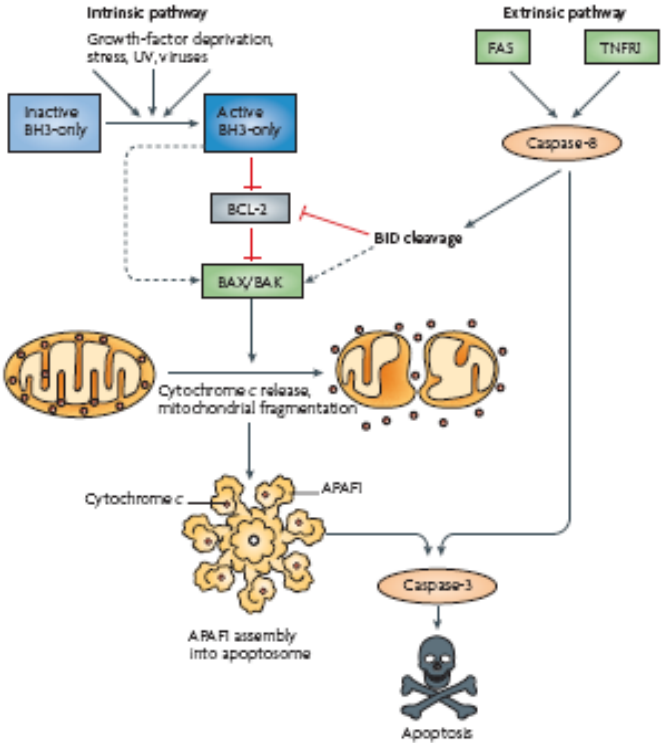


Figure 1.6 Scheme depicting the intrinsic and extrinsic pathways of apoptosis. Adapted from (Youle and Strasser, 2008).

1.4 Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) is a primary malignancy (cancer) of the liver. Most cases of HCC are secondary to either hepatitis (usually caused by hepatitis B or C virus infection) or cirrhosis (alcoholism being the most common cause of hepatic cirrhosis) (Kumar et al, 2003). HCC is the fifth most common cancer worldwide and the most common form of liver cancer in adults, being responsible for 80% of all primary liver tumors. HCC is highly resistant to available chemotherapeutic agents, resulting in a 5-year relative survival rate of less than 7% (Avila et al., 2006). Surgical hepatic resection may provide a chance of cure, but the prognosis of HCC patients remains poor (Poon et al., 1999). During the tumorigenesis of HCC, many cellular signaling pathways are altered, including the Wnt/β-catenin pathway, the receptor tyrosine kinase pathway, the NF-κB pathway, and the JAK/STAT pathway (Avila et al., 2006). A number of angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are also implicated in angiogenesis of HCC (Mise et al., 1996; Poon et al., 2001; Yamaguchi et al., 2006).

1.5 Hepatocyte proliferation

Hepatocyte proliferation plays a crucial role in liver regeneration. The most widely used model for the study of liver regeneration is partial hepatectomy (PH) introduced by Higgins and Anderson in 1931. A simple operation (PH) removes two-thirds of the liver of a rat or mouse, without damaging the lobes left behind and causing almost no liver tissue injury (Michalopoulos and DeFrances, 1997). Liver regeneration after PH is carried out by proliferation of all the existing cell types in the remnant liver including hepatocytes, endothelial cells, Kupffer cells, stellate cells, and oval cells (a population of periportal cuboidal cells with ovoid nuclei believed to represent liver stem cells), among which about 60% are hepatocytes (Pahlavan et al., 2006).

Generally, liver regeneration includes three steps: (1) initiation or priming, (2) proliferation, and (3) termination. The initiation step is characterized by priming of quiescent hepatocytes by cytokines such as tumor necrosis factor α (TNFα) and interleukin-6 (IL-6). Within a few hours after PH, a network of cytokines, either *de novo* synthesized or gut derived, activate hepatic non-parenchymal cells, leading to increased production of TNFα and IL-6. These cytokines then activate nuclear factor kappa B (NF-κB), signal transducer and activator of transcription 3 (STAT3), and possibly CAAT/Enhancer Binding Protein β (C/EBPβ), all of which are important hepatic transcription factors regulating primary growth responses in the liver. The activation of these transcription factors results in increased responsiveness of hepatocytes to growth factors and competence for replication (Fausto et al., 2006;

Pahlavan et al., 2006). During the proliferation phase, hepatocytes enter the G1 phase of the cell cycle, and are stimulated by numerous growth factors including hepatocyte growth factor (HGF), transforming growth factor α (TGFα), and epithelial growth factor (EGF). These hepatic mitogens together drive hepatocytes to override the G1/S checkpoint and progress into DNA synthesis (Pahlavan et al., 2006). Liver regeneration is a tightly regulated process. After progression of regeneration, a stop signal is sent to keep the regenerating liver at an appropriate functional size. Transforming growth factor β (TGFβ) has been shown to counteract the growth-promoting effect of other growth factors and regulate the termination of liver regeneration (Pahlavan et al., 2006).

STATEMENT OF PURPOSE

Retinoids, natural and synthetic derivatives of vitamin A, have long been recognized as essential nutrients for life. Clinically, retinoids are used to treat cancers and dermatologic disorders. Some retinoids have been reported to inhibit growth, induce differentiation and/or apoptosis in cancer cells. However, two large clinical trials, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study and the Beta-Carotene and Retinol Efficacy Trial (CARET) Study, which were designed to test whether β-carotene and vitamin A (retinol) could prevent lung cancer, respectively, actually revealed that long-term usage of vitamin A supplements increased the risk of lung cancer (1994; Albanes et al., 1996; Goodman et al., 2004; Virtamo et al., 2003). Moreover, all-trans retinoic acid is found to stimulate hepatocyte proliferation in mice (Ledda-Columbano et al., 2004). It is thus critical to determine the molecular basis responsible for these opposing effects of retinoids observed in experimental studies and clinical trials. Therefore, **the overall goal of this dissertation is to understand the molecular mechanisms underlying retinoid-induced apoptosis and proliferation of hepatocytes.**

Retinoids exert their biological effects by activating the nuclear receptors retinoic acid receptor (RAR) and retinoid X receptor (RXR). Both nuclear receptors consist of α, β, and γ subtypes. RAR and RXR can form heterodimers (RXR/RAR) and bind to the retinoic acid response element (RARE) in the promoter region of target genes to regulate transcription. Different retinoids may preferentially bind to

specific nuclear receptors and thus cause distinct effects. Therefore, the central hypothesis of this dissertation is that the **apoptotic or proliferative effect of certain retinoids in specific cellular contexts is mediated through activation of different nuclear receptors**.

Hypothesis 1:

Fenretinide induces apoptosis of HCC cells through activation of nuclear receptors RARβ and Nur77.

- **Specific Aim 1a:** Examine the susceptibility of HCC cells (Huh-7 and HepG2) to fenretinide-induced apoptosis.
- **Specific Aim 1b:** Determine the role of RARβ in the differential susceptibility of HCC cells to fenretinide-induced apoptosis.
- **Specific Aim 1c:** Determine the role of orphan nuclear receptor Nur77 in the differential susceptibility of HCC cells to fenretinide-induced apoptosis.

Hypothesis 2:

The proliferative effect of 13-cis retinoic acid in Hep3B cells is mediated through activation of alternative nuclear receptors.

- **Specific aim 2a:** Examine the proliferative effect of 13-cis retinoic acid in Hep3B cells and mouse livers.
- **Specific aim 2b:** Determine the ratio between cellular binding proteins FABP5 and CRABP II in Hep3B cells and mouse livers.

- **Specific aim 2c:** Examine the roles of the nuclear receptors PPARβ/δ pathway in 13-cis retinoic acid-induced proliferation of Hep3B cells.

Overall significance

Retinoids are used as food supplements, nutrients, and medicine for diseases including dermatological disorders and cancer. Several earlier clinical trials had suggested that retinoids might be used for cancer treatment and prevention. A better understanding of the molecular mechanisms of retinoids action will provide a rationale for the current clinical application of retinoids and broaden their therapeutic potentials. However, due to the diversity of retinoids and the receptors they can activate, the biological effects of retinoids are very versatile. Some retinoids show promising anti-tumor effects, while others are capable of stimulating cellular proliferation. The overall goal of this dissertation is to determine and characterize the different mechanisms responsible for retinoid-induced apoptosis and proliferation in the setting of liver cells.

(1) Retinoids.

A panel of thirteen retinoids was screened on three human HCC cell lines for induction of either apoptosis or proliferation. The retinoids examined include both natural and synthetic carotenoids and retinoids. The carotenoids tested were: β-carotene, lycopene, and lutein. The retinoids included were 9-cis retinal, 13-cis retinol,

13-cis retinal, 13-cis retinoic acid, all-trans retinyl palmitate, retinol acetate, and fenretinide. There are three receptor specific retinoids: 9-cis retinoic acid (ligand for RAR and RXR), all-trans retinoic acid (ligand for RAR) and 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) (a synthetic ligand for RAR). Among the thirteen retinoids, fenretinide and TTNPB are synthetic ones. The panel of retinoids used in this dissertation covers most commercially available retinoids, thus it yields a comprehensive screening results of retinoid-induced effects on human HCC cells.

(2) *in vitro* and *in vivo* models.

The *in vitro* model used in this dissertation to evaluate retinoid effect included three human HCC cell lines (Huh-7, HepG2, and Hep3B). The three HCC cell lines are most widely used in HCC-related laboratory research, and much experimental data has been generated from these cell lines. The *in vivo* model used in this dissertation was the retinoid-treated mouse model. The *in vivo* model was used to evaluate the proliferative effect of 13-cis retinoic acid in mouse livers. The *in vivo* model is also widely used, and the experimental procedure (gastric gavage) is well established with human relevance. More importantly, the data generated from these systems were considered relevant, accurate and convincing. Thus, the *in vitro and in vivo* effects of retinoids and the corresponding mechanisms were appropriately addressed.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Reagents. The retinoids used in this study are grouped into three categories: (1) carotenoids including β-carotene, lycopene, and lutein; (2) classic retinoids including all-trans retinol palmitate, retinol acetate, 9-cis retinaldehyde, 13-cis retinol, 13-cis retinaldehyde, 13-cis retinoic acid, and fenretinide; (3) receptor-specific retinoids including all-trans retinoic acid (ligand for RAR), 9-cis retinoic acid (ligand for both RAR and RXR), and TTNPB (4-(E-2-[5,6,7,8-tet-rahydro-5,5,8,8-tetramethyl-2-naphthalenyl]-1-propenyl) benzoic acid) (ligand for RAR). β-Carotene, lycopene, all-trans retinol palmitate, 9-cis retinaldehyde, 13-cis retinol, 13-cis retinoic acid, fenretinide, all-trans retinoic acid, 9-cis retinoic acid, and TTNPB were purchased from Sigma-Aldrich (St. Louis, MO). Lutein was purchased from US Biological (Swampscott, MA). Retinol acetate and 13-cis retinaldehyde were purchased from Toronto Research Chemicals (North York, Canada). Retinoids were dissolved in dimethyl sulfoxide (DMSO) at 10 mM as the stock solution and stored at -80°C. Retinoids were diluted with serum-free medium to a 10 μM final concentration immediately before use. The final concentration of DMSO in the culture medium was 0.1% in all treatments. Because retinoids are light sensitive, all retinoid treatments were conducted under dim light. Akt 1/2 inhibitor (1,3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one trifluoroacetate salt hydrate), and BrdU (5-bromo-2'-deoxyuridine) were purchased from Sigma-Aldrich (St. Louis, MO). Akt 1/2 inhibitor was dissolved in DMSO and the stock solution (0.5 mM) was stored at -20°C and diluted into 0.1, 0.2, and 0.5 μM with serum-free medium immediately

before use. BrdU was dissolved to 1mg/mL with water. Antibodies for Caspase-3, cleaved Caspase-3, Cyclin D1, D3, CDK-4, CDK-6, p21, p27, PDK-1, p-Akt (Thr308), p-Akt (Thr473), pan-Akt were purchased from Cell Signaling (Beverly, MA). Rabbit polyclonal antibodies for Nur77 and Poly ADP-ribose polymerase (PARP) were purchased from Santa Cruz (Santa Cruz, CA). Rabbit polyclonal antibody for Porin, PPARβ, and mouse monoclonal antibody for Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) were purchased from Abcam (Cambridge, MA).

2.2 Cell culture. Huh-7 cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) and HepG2 and Hep3B cells were cultured in Minimum Essential Medium (Mediatech, Herndon, VA). The media were supplemented with 10% fetal calf serum (FBS) (Atlanta Biologicals, Lawrenceville, GA). Cells were cultured at 37°C in 5% CO_2 atmosphere with a relative humidity of 95%. Cells were plated at approximately $1x10^6$ cells per T-25 flask, $5x10^5$ per well of 6-well plates, $1x10^5$ per well of 24-well plates, or $1x10^4$ per well of 96-well plates 12-16 hours prior to the treatments. The next day cells were rinsed with PBS to remove residual of FBS and incubated with individual retinoids (10 μM), Akt inhibitor, or any denoted combination in serum-free media for the indicated time. Fresh medium containing individual retinoids/chemical was provided every 24 hours whenever needed. Cell viability was determined by trypan blue exclusion counting with a hemocytometer according to the manufacturer's instruction. Every sample was counted in triplicates.

2.3 Immunostaining. Following treatment, air-dried cells were fixed with PBS containing 1% paraformaldehyde for 15 minutes at room temperature. After rinsing with PBS containing 0.2% Triton X100 for three times (5 minutes each time), cells are incubated with PBS containing 0.2% Triton X100 and 5% normal goat serum for 30 minutes at room temperature to block endogenous nonspecific binding sites. Cells were then incubated with primary antibody for protein of interest (diluted at 1:100, in PBS containing 0.2% Triton X100 and 1% normal goat serum) for 1 hour at 37°C or at 4°C overnight in a humidified chamber. After washed with PBS containing 0.2% Triton X100 and 1% normal goat serum for three times (20 minutes each time), cells were incubated with FITC-conjugated secondary antibody (diluted at 1:400, in PBS containing 0.2% Triton X100 and 1% normal goat serum) for 1 hour at 37°C or 3 hours at room temperature in a humidified chamber. After washed with PBS for three times (20 minutes each time), cells were mounted with VECTASHIELD Mounting Medium containing DAPI and read under fluorescence or confocal microscope. The mounted slides can be stored at -20°C for extended periods in necessary.

2.4 In vitro BrdU incorporation assay. Hep3B cells were incubated with serum-free MEM containing DMSO or 13-cis RA (10 μM) in flat bottom 96-well plates for indicated time periods. At the end of treatments, cells were incubated with fresh MEM containing 10 μM BrdU for 2-4 hours at 37 °C to allow the pyrimidine analogue BrdU to be incorporated into the newly synthesized DNA strands. The following BrdU immunostaining was performed using a Cell Proliferation ELISA BrdU (colorimetric) kit (Roche Applied Science, Indianapolis, IN) according to the

manufacturer's instruction. Plates were read with a microplate/ELISA reader at 370 nm (with a reference absorbance at 492 nm). BrdU incorporation index was reported by the normalized absorbance.

2.5 Mice, 13-cis RA gavage, and sample preparation. C57BL6 male mice (4-5 months old), were housed in steel microisolator cages at 22°C with a 12-hour/12-hour light/dark cycle. Food and water were provided *ad libitum* throughout the entire period. Corn oil or 13-cis RA (10 mg/Kg body weight) was given by gastric gavage once a day for 1, 2, 5, and 10 days. Body weight was monitored everyday. Livers were harvested and weighed at the end of treatments, and aliquots of the livers were stored at -80°C until use.

2.6 In vivo BrdU incorporation. During retinoid treatment, mice received BrdU (1mg/mL, Sigma) in drinking water. At the indicated time points, livers were harvested and fixed in formalin, embedded in paraffin, and processed into 5-micron sections. *In vivo* BrdU incorporation was determined using a BD Pharmingen BrdU In-Situ Detection Kit (BD Biosciences, San Jose, CA) and VECTASTAIN ABC Kit (Vector Laboratories, Burlingame, CA) according to the manufacturers' instructions.

2.7 Subcellular fraction isolation. Trypsinized cells ($1x10^7$) were collected by centrifugation at 1000 rpm for 5 minutes. Cell pellets were resuspended with 5.5 mL of cold RSB buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, supplemented with protease and phosphatase inhibitors) and incubated on ice for 90 minutes. Cells were then lysed in a Dounce homogenizer for 30 minutes and the

lysate was monitored by microscopic observation. Then the lysate was mixed with 4 mL 2.5x MSB buffer (525 mM Mannitol; 175 mM Sucrose; 12.5 mM Tris-HCl, pH 7.5; 2.5 mM EDTA, pH 7.5) to make a final 1x MSB buffer in the lysate. The lysate was centrifuged at 1,300 xg for 5 minutes at 4°C for two consecutive times and the pellets were combined as the nuclear fraction. The supernatant was then centrifuged at 17,000 xg for 15 minutes at 4°C and the subsequent supernatant represented cytosol fraction and the pellets represented crude mitochondria fraction. All fractions were aliquoted and stored at -20°C until use.

2.8 Western blotting and antibodies. Cells from indicated treatments were collected and lysed with lysis buffer (50 mM Tris·Cl, pH 7.4; 150 mM NaCl; 2 mM EDTA; 0.1% SDS; 1% (V/V) NP-40 with protease and phosphatase inhibitors (Pierce, Rockford, IL)). Equal amounts of lysates (50 μg total protein) were separated by SDS-PAGE and electroblotted onto PVDF membrane (Bio-Rad, Hercules, CA). The membranes were first incubated with PBS supplemented with 0.1% Tween 20 and 5% nonfat dry milk (PBST-milk) for 1 hour at room temperature to block nonspecific binding sites on the membrane. Immunostaining was performed by incubating the membranes with primary antibodies for Caspase-3, cleaved Caspase-3, Cyclin D1, D3, CDK-4, CDK-6, p21, p27, PDK-1, p-Akt (Thr308), p-Akt (Thr473), pan-Akt, PPARβ, GAPDH, or Nur77, PARP, Porin, β-actin (Santa Cruz, Santa Cruz, CA) in PBST-milk overnight at 4°C. After three washes in PBS supplemented with 0.1% Tween 20 (PBST) (15 minutes each time), membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at room

temperature in PBST-milk followed by three washes (15 minutes each time). Signal was detected using the ECL system SuperSignal West Pico Chemiluminescent Substrates (Pierce, Rockford, IL).

2.9 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Cells ($5x10^4$) were plated into chamber slides (BD, Franklin Lakes, NJ) in medium supplemented with 10% FBS and cultured overnight to attach. The next morning, cells were washed with PBS and incubated with fenretinide (10 μM) in serum-free medium for 24 hours followed by TUNEL staining using an *in situ* cell death detection kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instruction. The stained slides were examined under light microscope and TUNEL staining positive cells were recorded per field.

2.10 Flow cytometry. Cells ($1x10^6$) were plated into T-25 flasks in medium supplemented with 10% FBS and cultured overnight to attach. The next morning, cells were washed with PBS and incubated with fenretinide (10 μM) in serum-free medium for 24, 48, or 72 hours. Medium containing fresh fenretinide were provided every 24 hours. Attached cells were collected at each time point and processed for Annexin V-FITC and propidium iodide (PI) double staining using Annexin V-FITC Apoptosis Detection kit (BD Biosciences, San Jose, CA) according to the manufacturer's instruction. For cell cycle distribution analysis, the trypsinazed cells from each treatment were collected by centrifuge at 500 rpm for 5 minutes and the pellets were resuspended in 0.5 mL 0.9% NaCl. The suspended cells were gently

vortexed and 1.25 mL cold 90% ethanol was added into each sample dropwise. Samples were held at room temperature for 30 minutes and followed by spinning down the cells at 500 rpm for 5 minutes. The pellets were vortexed and resuspended with 0.5 mL staining solution (50 μg/mL PI and 0.2 mg/mL in PBS). Samples were gently vortexed and incubated at 37 °C in dark for 30 minutes then analyzed for cell cycle distribution on a Fluorescence Activated Cell Sorter (FACS) (BD Biosciences, San Jose, CA).

2.11 Total RNA preparation. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. RNA was quantified and assessed for purity on a UV spectrophotometer.

2.12 Reverse transcription and quantitative real-time PCR. Total RNA (1 μg) was reverse-transcribed with oligo (dT) primer and MMLV RT reverse transcriptase (Invitrogen, Carlsbad, CA) for cDNA synthesis. cDNA corresponding to 32 ng total RNA was used as the template in a 20 μL real-time PCR reaction with the ABI TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and the appropriate primer pair and Taqman probe. All primer pairs and Taqman probes were designed with Primer Express software v2.0 (Table 3.1 and 5.1). Real-time PCR was conducted using the ABI Prism 7300 real-time PCR system (Applied Biosystems, Foster City, CA). The quantification analysis for target gene expression was performed using the relative quantification comparative CT method (Livak and Schmittgen, 2001).

2.13 Transient transfection luciferase reporter assay. Cells ($1x10^5$ cells per well) were cultured overnight in 24-well plates and then transfected with different plasmids using Lipofectamine (Invitrogen, CA) according to the manufacturer's instruction. A luciferase reporter construct harboring a retinoic acid response element (RARE/DR5) or peroxisome proliferator response element (PPRE) (300 ng) and expression plasmids for RXRα and RARβ or RXRα and PPARβ (50 ng) (provided by Dr. Ronald Evans, Salk Institute, CA) were used for co-transfection. A Renilla luciferase expression plasmid (10 ng) was also included in co-transfection for normalization of transfection efficiency. Cells were then treated with either DMSO or fenretinide (10 μM) for 48 hours. Fresh medium containing fenretinide was provided every 24 hours. After 48 hours, cells were harvested, lysed with passive lysis buffer, and firefly and renilla luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) in a luminometer.

2.14 Transfection. cDNAs of hPPARβ from Hep3B cells were cloned into the pZsGreen1-N1 vector (Clontech, Mountain View, CA). Hep3B cells were seeded into 6-well plates ($5x10^5$/well) 16 hours prior to transfection. pZsGreen1-N1-hPPARβ or empty vector (1 μg each) was transfected into Hep3B cells using Lipofectamine (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. Cells were lysed to extract total protein 48 hours post-transfection to confirm PPARβ overexpression by western blotting. For co-transfection empty vector, RXRα and/or PPARβ expression constructs (80 ng of each construct per well of 96-well plates) were included for 24 hours followed by 13-cis RA treatment and BrdU incorporation assay.

2.15 Chromatin immunoprecipitation (ChIP) assay. ChIP was performed using the ChIP assay kit from Upstate (Charlottesville, VA) and antibodies specific for RARβ (1:600, Santa Cruz, Santa Cruz, CA). Control ChIP was performed using a normal rabbit IgG (Santa Cruz, Santa Cruz, CA). The immunoprecipitated DNA fragments were amplified by PCR with primer pairs encompassing the proximal RARE of human RARβ or CYP26A1 gene (RARβ: sense 5'-TGGGTCATTTGAAGGTTAG-3', antisense 5'-GTTCTCGGCATCCCAGTC-3'; CYP26A1: sense 5'-CCGCAATTAAAGATGAACT-3', antisense 5'-TACAGGTCCCAGAGCTTGAT-3').

2.16 siRNA transfection. Scramble siRNA and pre-designed siRNA for human gene of interest were purchased from Ambion (Austin, TX). Huh-7 cells were transfected with siRNA (10 nM per $1x10^5$ cells) using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. Trypsinized cells ($1x10^5$cells/mL) were incubated with diluted siRNA/ Lipofectamine™ RNAiMAX Transfection Reagent complex (12 pmol siRNA/6 μL transfection reagent) in serum-free medium for 20 minutes followed by incubation at 37°C for 48 hours. Cells were then harvested 48 hours post-transfection for evaluation of RARβ knockdown efficiency by real-time PCR.

2.17 Statistical analysis. Data are presented as mean ± S.E. Statistical analysis was performed using Student's *t*-test for two-group comparison or one-way ANOVA for three or more group comparisons. Significance was defined by $p < 0.05$.

CHAPTER THREE

Fenretinide-induced Apoptosis of Huh-7 Hepatocellular Carcinoma is Retinoic Acid Receptor β Dependent

Abstract

Retinoids are used to treat several types of cancer; however, their effects on liver cancer have not been fully characterized. To investigate the therapeutic potential of retinoids on human hepatocellular carcinoma (HCC), the present study evaluates the apoptotic effect of a panel of natural and synthetic retinoids in three human HCC cell lines as well as explores the underlying mechanisms. Our data revealed that fenretinide effectively induces apoptosis in Huh-7 and Hep3B cells. Gene expression analysis of nuclear receptor genes revealed that the basal and inducibility of retinoic acid receptor β (RARβ) expression positively correlate with the susceptibility of HCC cells to fenretinide treatment. Furthermore, fenretinide transactivates the RXRα/RARβ-mediated pathway and directly increases the transcriptional activity of RARβ as demonstrated by transactivation assay and chromatin immunoprecipitation (ChIP) assay. Knockdown of RARβ mRNA expression by siRNA significantly impairs fenretinide-induced apoptosis in Huh-7 cells. Our results demonstrate that fenretinide activates RARβ and induces RARβ-dependent apoptosis in Huh-7 cells. These findings suggest a novel role of RARβ as a tumor suppressor by mediating the signals of certain chemotherapeutic agents.

Introduction

Hepatocellular carcinoma (HCC), the primary malignancy of the liver, is the third most common cause of cancer-related mortality worldwide (Bruix et al. 2006). HCC is highly resistant to available chemotherapy, resulting in a 5-year relative survival rate of less than 7% (Avila et al. 2006). Thus, discovery of new and effective therapies against HCC is much needed.

Retinoids, the natural and synthetic derivates of vitamin A, has a long history in clinical application in addition to its roles as an essential nutrient. Historically, Egyptians used roasted ox liver, which is rich in vitamin A, to treat night blindness. Nowadays, physicians prescribe drugs containing retinoids to treat dermatological disorders and leukemia. Moreover, data from experimental animal models and preclinical, epidemiological, and clinical studies suggest that retinoids may also have chemopreventive and anticancer effect. The best example of retinoid anticancer effect is the retinoic acid (RA) differentiation therapy for acute promyelocytic leukemia (APL) (Fontana and Rishi 2002). The use of RA has changed the clinical course of APL from a highly lethal to a curable leukemia, therefore establishing the prototype of retinoid-based therapies and the rationale for the use of retinoids in the treatment and prevention of cancer (Clarke et al. 2004). In addition, retinoids have been used either alone or in combination with other chemotherapeutic agents to treat other types of cancer and precancerous lesions. The anti-proliferative effect of tamoxifen is synergistically enhanced when used in combination with retinoids (Herold et al. 2002).

Retinoids also show promising effects in adjuvant therapy for HCC (Gerard and Bleiberg 2004). However, the therapeutic potentials of retinoids against HCC have not been extensively investigated. In the present study, we initiated a comprehensive screening including most commercially available retinoids on three widely used human HCC cell lines for apoptosis induction. Agree with previous studies (You et al. 2001; Kim et al. 2002), we found that fenretinide (N-[4-hydroxyphenyl] retinamide or 4HPR) induces apoptosis in Hep3B cells. In addition, we found that fenretinide also effectively induces apoptosis in Huh-7 cells. In contrast, HepG2 cells are resistant to fenretinide treatment. To elucidate the mechanisms underlying the observed differential susceptibility, gene expression analysis of twelve nuclear receptor genes were assessed by real-time PCR. Our data strongly suggest that the susceptibility of HCC cells to fenretinide treatment is determined by the basal and the induced expression level of RARβ. Furthermore, we showed that fenretinide directly activates RARβ in Huh-7 cells. Finally, the RARβ-deficient Huh-7 cells exhibit marked reduction of fenretinide-induced apoptosis. Based on these findings, we conclude that, in Huh-7 cells, fenretinide directly activates RARβ and induces apoptosis in a RARβ-dependent manner.

Results

Fenretinide induces apoptosis in Huh-7 and Hep3B cells, but not in HepG2 cells. Several studies have shown that retinoids have anti-proliferation or apoptotic effects in certain cancer cells (Clarke et al., 2004). To assess the effect of retinoids on HCC cells, we examined cell viability and caspase-3 cleavage induced by individual retinoids in three human HCC cell lines (Figure 3.1). As an initial screening, 10 μM was used for all the tested retinoids. This dose might be high for certain retinoids, however, besides apoptosis, no obvious cytotoxicity was noted during the 3-day treatment. In Huh-7 cells, nine out of thirteen retinoids decreased cell viability, with fenretinide being the most effective one (79% decrease in cell number compared with DMSO treatment). Fenretinide also induced the strongest caspase-3 cleavage in detached Huh-7 cells and 9-cis retinoic acid caused a modest induction (Figure 3.1A). In HepG2 cells, although all retinoids examined significantly decreased cell viability, only 9-cis retinoic acid induced weak caspase-3 cleavage (Figure 3.1B). In Hep3B cells, six out of thirteen retinoids decreased cell viability, whereas another three retinoids increased cell number (Figure 3.1C). 9-cis retinoic acid, fenretinide, TTNPB, and lutein induced strong caspase-3 cleavage in Hep3B cells (Figure 3.1C). These findings indicate that fenretinide induces apoptosis in both Huh-7 and Hep3B cells, but not in HepG2 cells.

To further confirm the differential responses of Huh-7 and HepG2 cells to fenretinide, both cell lines were treated with fenretinide and assessed for caspase-3 cleavage, DNA double-strand breaks, and phosphatidylserine translocation in a time

course study. In Huh-7 cells, caspase-3 cleavage was detected at as early as 24 hours after treatment, and the induction was sustained at 48 and 72 hours. In HepG2 cells, however, even after 72 hours treatment, no obvious caspase-3 cleavage was detected (Figure 3.2A). DNA double-strand breaks, another hallmark of apoptosis, assessed by the TUNEL assay, were detected in Huh-7 cells after treatment (Figure 3.2B). In contrast, no significant increase in DNA double-strand breaks was detected in HepG2 cells after treatment (Figure 3.2B). Some background TUNEL staining was detected in HepG2 cells possibly due to the endogenous peroxidase activity. In addition, during apoptosis, membrane lipid phosphatidylserine translocates from the inner leaflet of the plasma membrane to the outer leaflet, resulting in loss of cell membrane asymmetry. Fenretinide induced phosphatidylserine translocation in Huh-7 cells in a time-dependent manner, reaching 17-fold after 72 hours (Figure 3.33A). However, fenretinide failed to induce such changes in HepG2 cells even after a 3-day treatment (Figure 3.3B). Taken together, these findings convincingly demonstrate that Huh-7 cells are susceptible to fenretinide-induced apoptosis, but HepG2 cells are resistant.

High basal and inducibility of RARβ gene expression in HCC cells is associated with their susceptibility to fenretinide-induced apoptosis. To determine whether nuclear receptors mediate the apoptotic effect of fenretinide in HCC cells, the basal mRNA levels of twelve nuclear receptors were assessed by real-time PCR (Figure 3.4A). Among the three cell lines, Huh-7 cells have the highest basal mRNA levels of RARβ, RXRα, and an orphan nuclear receptor Nurr1 (also known as NR4A2). Hep3B cells have the second highest mRNA levels of RARβ and Nurr1. In

contrast, in HepG2 cells, the basal mRNA level of RARβ is undetectable. On the other hand, in HepG2 cells, the basal mRNA levels of SXR (steroid and xenobiotic receptor) and CAR (constitutive androstane receptor), two xenobiotic sensors that mediate many xenobiotic responses, are the highest among the three cell clines.

The regulation of these twelve nuclear receptor genes by fenretinide were then evaluated in Huh-7 and HepG2 cells by real-time PCR. The induction fold was calculated by comparing the mRNA level of each nuclear receptor gene between DMSO and fenretinide treatment at each time point. Only those genes that showed marked changes in expression were presented. In Huh-7 cells, fenretinide caused a continuous induction of RARβ mRNA level (Figure 3.4B). After 48 hours of treatment, mRNA levels of RARα and γ were also highly induced in Huh-7 cells (Figure 3.4B). In contrast, a 9-fold induction of RARβ was detected 6 hours after fenretinide treatment in HepG2 cells, and then the induction dropped down to 3-5 fold later on. NOR1 (NR4A3) mRNA was induced 6-fold after 48 hours treatment in HepG2 cells (Figure 3.4C). Furthermore, by comparing the RARβ mRNA level between Huh-7 and HepG2 cells after fenretinide treatment, our data revealed a dramatic difference in RARβ mRNA level between these two cell lines (Figure 3.4D). Taken together, these data clearly depict a positive correlation between RARβ mRNA level and susceptibility to fenretinide-induced apoptosis, which suggests that RARβ may play an important role in mediating fenretinide-induced apoptosis in HCC cells.

Fenretinide activates RXRα/RARβ-mediated pathway. It is known that RARβ induces it own expression upon stimulation by RAR ligands (Bastien and

Rochette-Egly, 2004). Since fenretinide is a synthetic retinoid whether it can directly activate RARβ remains to be tested. So we examined whether fenretinide activates RARβ. We first tested whether fenretinide can activate the RXRα/RARβ-mediated pathway by transactivation assay (Figure 3.5). Fenretinide caused a marked induction of luciferase activity in Huh-7 cells (43-fold) and in CV-1 cells (13-fold) (Figure 3.5A and B). In contrast, fenretinide did not significantly increase luciferase activity in HepG2 cells (Figure 3.5C). These data indicate that fenretinide can transactivate RXRα/RARβ-mediated pathway in Huh-7 but not in HepG2 cells. As shown in Figure 3.4C, a modest induction of RARβ mRNA was seen in HepG2 cells 6 hours after fenretinide treatment, but no sustained induction at 48 hours. Consistently, no significant increase in luciferase activity, which was measured 48 hours after fenretinide treatment, was detected in HepG2 cells.

Fenretinide increases the transcriptional activity of RARβ in Huh-7 cells. The most direct evidence of RARβ activation is the increased binding of RARβ to the response elements in retinoid target genes. It is known that RARβ binds to the RARE in its own promoter upon RA treatment (Bastien and Rochette-Egly, 2004; Geisen et al., 1997). One of the classic RAREs, a 5-bp spaced direct repeat (DR5), was found in the promoter of RARβ (Bastien and Rochette-Egly, 2004). Another well established retinoid target gene is cytochrome P450 26A1 (CYP26A1), an enzyme that catalyzes the breakdown of retinoic acid to more polar metabolites (Bastien and Rochette-Egly, 2004). Two RAREs have been found in the promoter of CYP26A1, one in the proximal region and the other in the distal region (Loudig et al., 2005). Using

chromatin immunoprecipitation (ChIP) assay, the direct binding of RARβ to the RAREs in RARβ and CYP26A1 upon fenretinide treatment was revealed. Fenretinide increased the binding (14-fold) of RARβ to its own promoter compared with DMSO treatment (Figure 3.6A). An even higher increase (27-fold) of RARβ binding to the CYP26A1 promoter was also noted (Figure 3.6B). Together, these results demonstrate that fenretinide directly activates RARβ.

Knockdown of RARβ mRNA expression by siRNA reduces fenretinide-induced apoptosis in Huh-7 cells. To determine the role of RARβ in mediating fenretinide-induced apoptosis, the endogenous RARβ mRNA expression in Huh-7 cells was knocked down using siRNA. The knockdown efficiency of RARβ by three sequence-independent siRNA oligonucleotides was evaluated by real-time PCR. Three siRNAs silenced RARβ gene expression to different extents, the most efficient knockdown being 86% (siRNA #4124) compared with scramble siRNA, followed by 81% (siRNA #3935) and 68% (siRNA #4030) (Figure 3.7A). The apoptotic effect of fenretinide was then evaluated in these RARβ-deficient cells. Our results showed that DNA double-strand breaks induced by fenretinide were markedly reduced in RARβ-deficient Huh-7 cells (Figure 3.7B). Consistent with RARβ knockdown level, the greatest reduction of apoptosis (88.6%) was seen in the cells with the lowest endogenous RARβ mRNA level (cells transfected by siRNA #4124 with 86% knockdown of RARβ mRNA), followed by 83.1% in cells transfected by siRNA # 3935 with 81% knockdown of RARβ mRNA, and 70.7% in cells transfected by

siRNA # 4030 with 68% knockdown of RARβ mRNA. These data clearly demonstrate that fenretinide-induced apoptosis of Huh-7 cells is RARβ dependent.

Table 3.1: Real-time PCR primers and probes used in this study.

Target Gene	Gene Bank Acession NO.		Primer Sequence (5'-3') Sequences are shown for forward (F) and reverse (R) primers	Probe Sequence (5'-3') with modification of 5' FAM / 3' BHQ1
hRARα	NM_000964	F	GACAAGTCCTCAGGCTACCACTATG	CTGCAAGGGCTTCTTCCGCCG
		R	GTACACCATGTTCTTCTGGATGCT	
hRARβ	NM_000965	F	TCTCAGTGCCATCTGCTTAATCTG	CCAGGACCTTGAGGAACCGACAAAAG
		R	CCAGCAATGGTTCTTGTAGCTTATC	
hRARγ	NM_000966	F	GCTGCAAGGGCTTCTTTCG	CGAAGCATCCAGAAGAACATGGTGTAC
		R	CAGTTTTTGTCGCGGTGACA	
hRXRα	NM_002957	F	TCCTTGGAGGCCTACTGCAA	CAGCCGGGAAGGTTCGCTAAGC
		R	GCATTTGAGCCCGATGGA	
hRXRβ	NM_021976	F	AGCAGCAGGGACGGTTTG	AAGCTGCTGCTACGTCTTCCTGCCC
		R	GCTCTAGACACTTAAGGCCAATGG	
hRXRγ	NM_006917	F	ACCTTGGAGGACCAGGTCATT	TGCTTCGGGCAGGGTGGAATG
		R	GGAGAAAGAGGCAATCAGCAA	
hNur77	NM_002135	F	AGCATTATGGTGTCCGCACAT	TGAGGGCTGCAAGGGCTTCTTCAA
		R	TTGGCGTTTTTCTGCACTGT	
hNurr1	NM_006186	F	TGGGATGGTCAAAGAAGTGGTT	TTTAAAAGGCCGGAGAGGTCGTTTGC
		R	TGGGCTCTTCGGTTTCGA	
hNOR1	NM_006981	F	ATGCCCTTGTCCGAGCTTT	AACACCCAGAGATCTTGATTATTCCAGA
		R	AGCCTGGTCAGTGGGACAGT	
hCAR	NM_005122	F	CACATGGGCACCATGTTTGA	TTTGTGCAGTTTAGGCCTCCAGCTCATCT
		R	AAGGGCTGGTGATGGATGAA	
SXR	NM_003889	F	TCCCCAAATCTGCCGTGTAT	ACAAGGCCACTGGCTATCACTTCAATGTCA
		R	AGCCCTTGCATCCTTCACAT	
hPPARα	NM_005036	F	AGCTCCCGTATCTTTTGTTATGTTG	GTCTGCGCTCCAGAGAGCATCTACTGTCA
		R	TCGATCCGCAGGGTGACT	
hβ-Actin	NM_001101	F	CCTGGCACCCAGCACAAT	ATCAAGATCATTGCTCCTCCTGAGCGC
		R	GCCGATCCACACGGAGTACT	

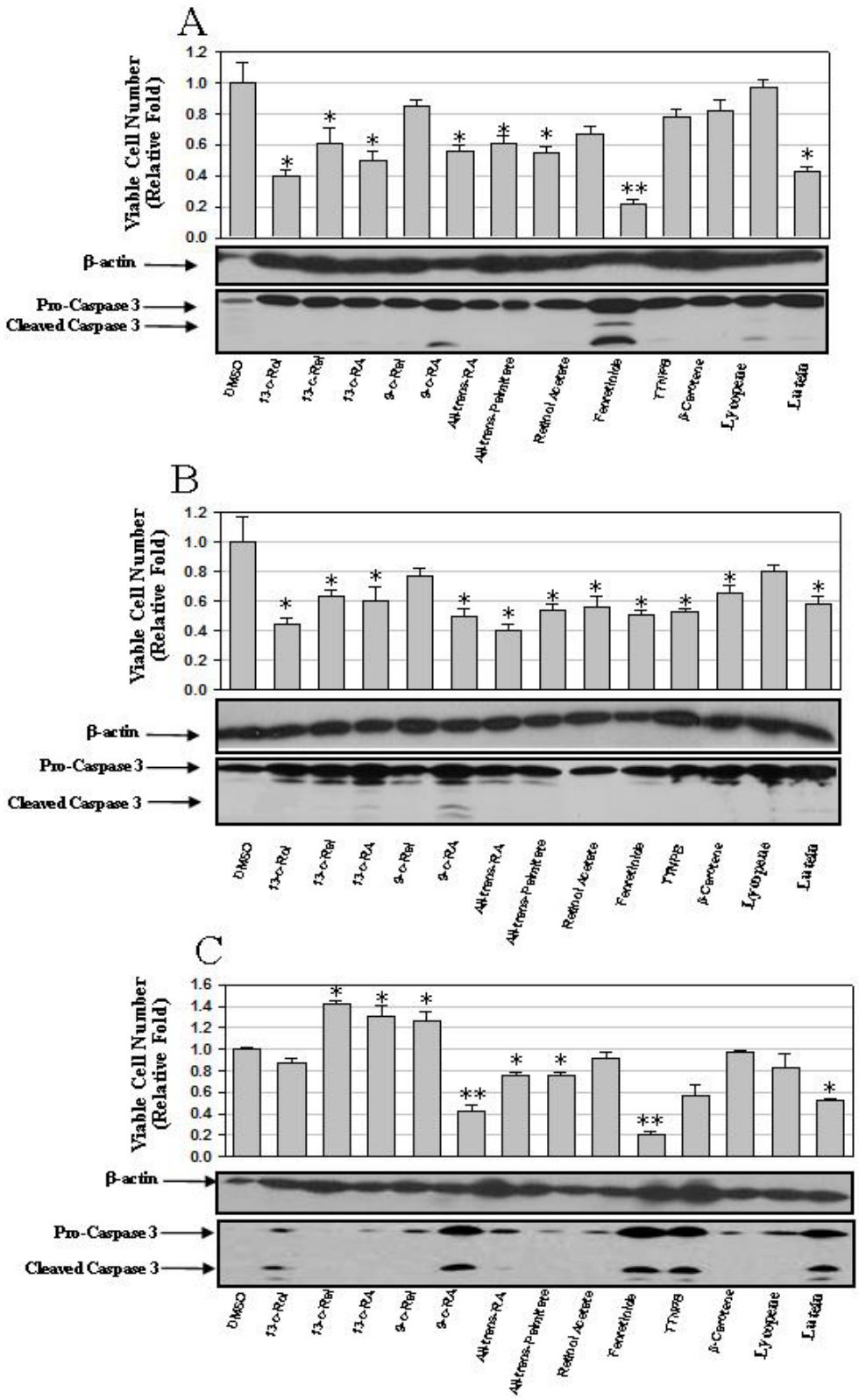

A
Viable Cell Number (Relative Fold)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
β-actin
Pro-Caspase 3
Cleaved Caspase 3
DMSO
13-c-Rol
13-c-Rel
13-c-RA
9-c-Rel
9-c-RA
All-trans-RA
All-trans-Palmitate
Retinol Acetate
Fenretinide
TTNPB
β-Carotene
Lycopene
Lutein
B
Viable Cell Number (Relative Fold)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
β-actin
Pro-Caspase 3
Cleaved Caspase 3
DMSO
13-c-Rol
13-c-Rel
13-c-RA
9-c-Rel
9-c-RA
All-trans-RA
All-trans-Palmitate
Retinol Acetate
Fenretinide
TTNPB
β-Carotene
Lycopene
Lutein
C
Viable Cell Number (Relative Fold)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
β-actin
Pro-Caspase 3
Cleaved Caspase 3
DMSO
13-c-Rol
13-c-Rel
13-c-RA
9-c-Rel
9-c-RA
All-trans-RA
All-trans-Palmitate
Retinol Acetate
Fenretinide
TTNPB
β-Carotene
Lycopene
Lutein

Figure 3.1: Retinoids decrease cell viability and induce caspase-3 cleavage in HCC cells.

Huh-7 (A), HepG2 (B), and Hep3B cells (C) were incubated in serum-free medium containing individual retinoids (10 μM) for three days. Viable cells were counted and presented as a relative fold of DMSO treatment. Caspase-3 cleavage in detached cells was determined by Western blot. (* $p < 0.05$, ** $p < 0.01$ compared to DMSO treatment). Data were presented as mean ± S.E.M. Cells were counted in triplicates. Western blot results shown were representative results of two independent experiments.

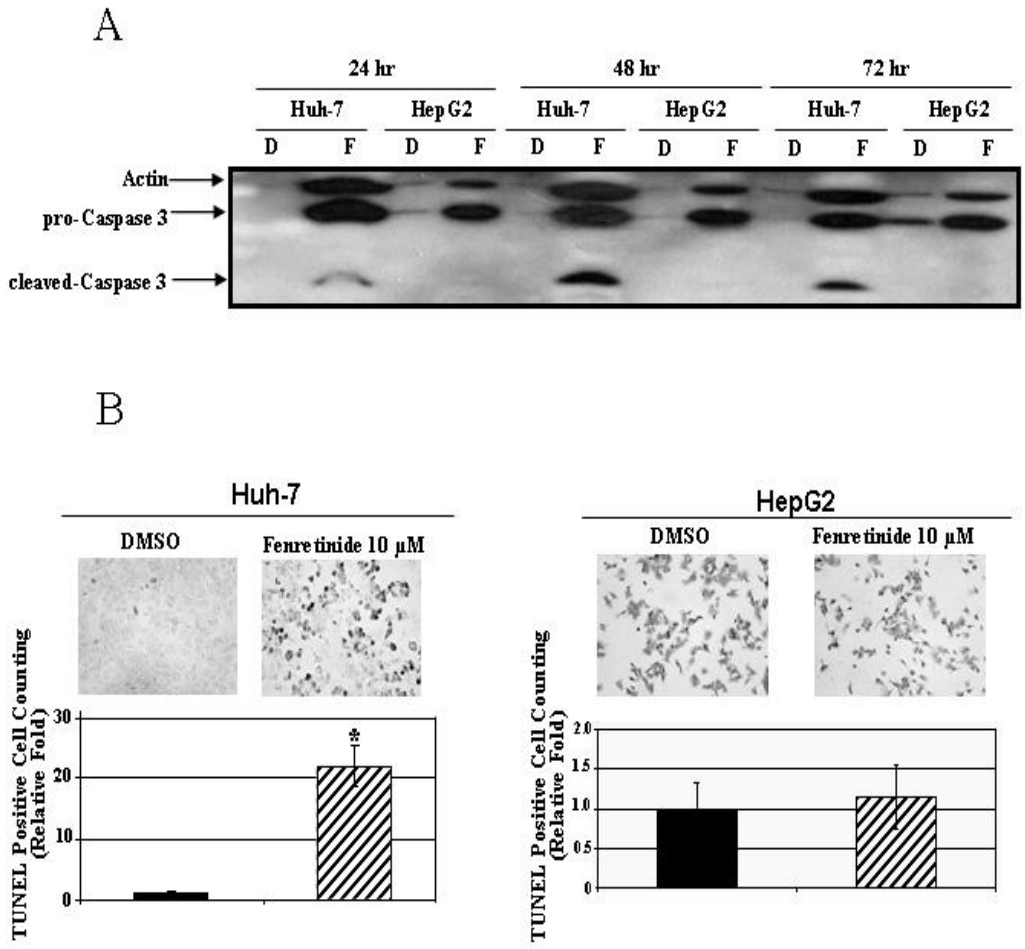

A
24 hr
48 hr
72 hr
Huh-7
HepG2
Huh-7
HepG2
Huh-7
HepG2
D F D F D F D F D F D F
Actin
pro-Caspase 3
cleaved-Caspase 3
B
Huh-7
DMSO
Fenretinide 10 μM
TUNEL Positive Cell Counting (Relative Fold)
30
20
10
0
*
HepG2
DMSO
Fenretinide 10 μM
TUNEL Positive Cell Counting (Relative Fold)
2.0
1.5
1.0
0.5
0

Figure 3.2: Fenretinide causes caspase-3 cleavage and DNA double-strand breaks in Huh-7 cells but not in HepG2 cells.

Huh-7 and HepG2 cells were treated with either DMSO or fenretinide and analysed for caspase-3 cleavage by Western blot (A) and DNA double-strand breaks by TUNEL assay (B). TUNEL positive counting was presented as a relative fold of DMSO treatment, and the staining was representative result of two independent experiments. Data were shown as mean ± S.E.M. (* $p < 0.05$, ** $p < 0.01$ compared with DMSO treatment).

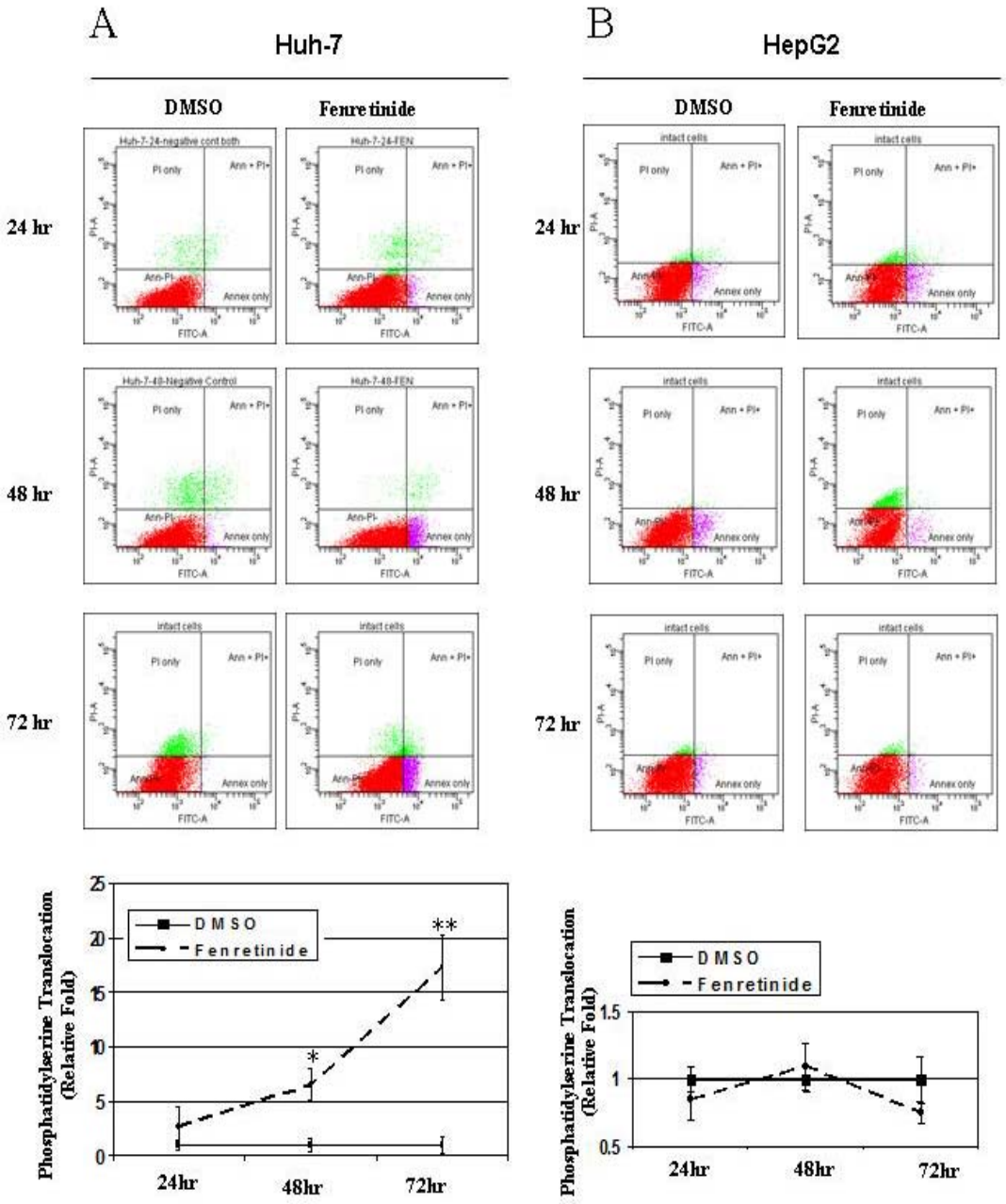

A
Huh-7
B
HepG2
DMSO
Fenretinide
24 hr
48 hr
72 hr
PI only
Ann + PI+
Ann-PI-
Annex only
PI-A
FITC-A
Phosphatidylserine Translocation (Relative Fold)
DMSO
Fenretinide
24hr
48hr
72hr
*
**

Figure 3.3: Fenretinide causes phosphatidylserine translocation in Huh-7 cells but not in HepG2 cells.

Huh-7 (A) and HepG2 cells (B) were treated with fenretinide and analysed for phosphatidylserine translocation by flow cytometry analysis. The percentage of cells with phosphatidylserine translocation was presented as a relative fold of DMSO treatment. Flow cytometry data were representative results from three independent experiments. Data were shown as mean ± S.E.M. (* $p < 0.05$, ** $p < 0.01$ compared with DMSO treatment).

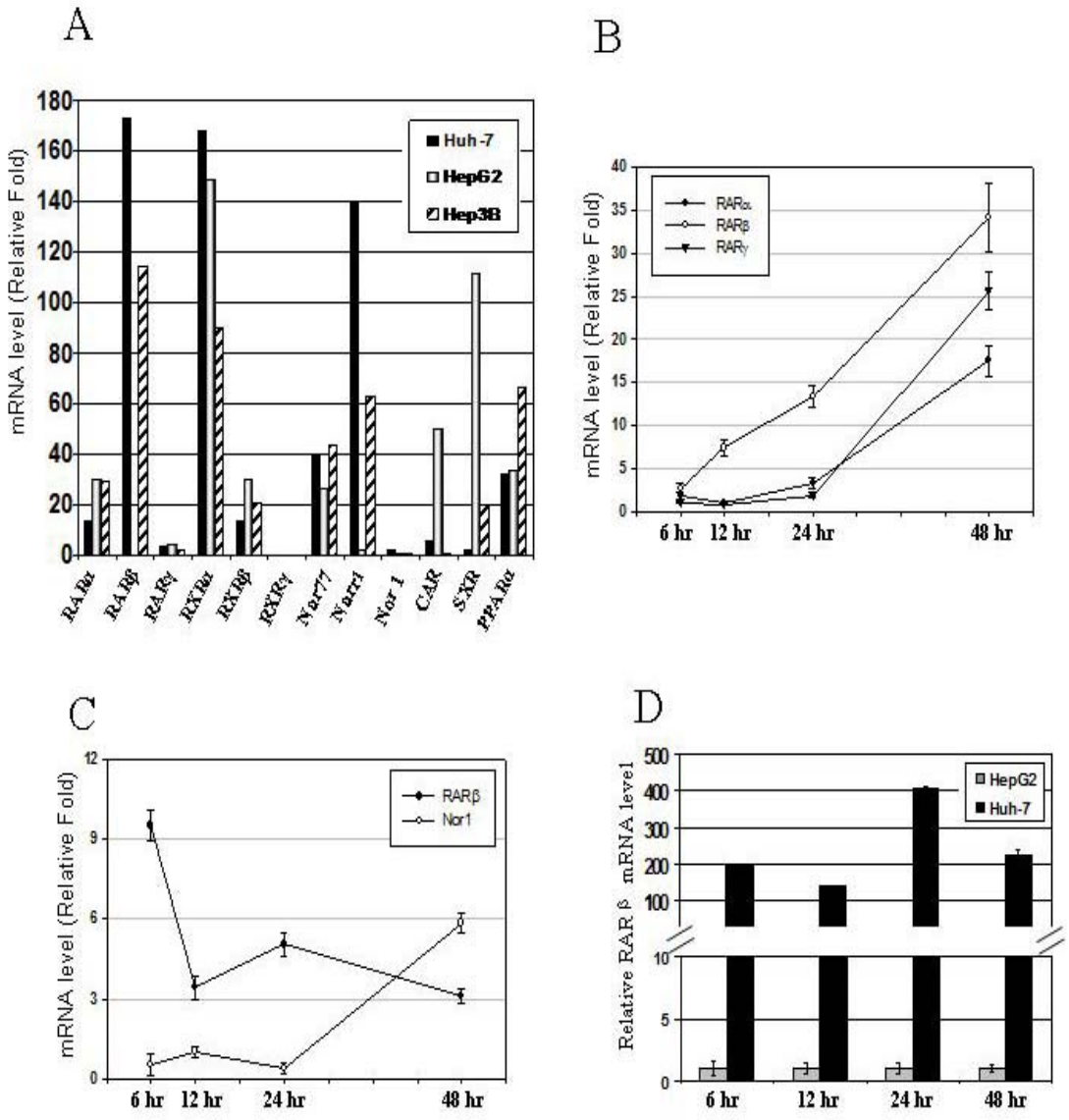

A
B
C
D
mRNA level (Relative Fold)
Huh-7
HepG2
Hep3B
RARα
RARβ
RARγ
6 hr
12 hr
24 hr
48 hr
Nor1
Relative RAR β mRNA level

Figure 3.4: RARβ expression in HCC cells positively correlates with the susceptibility to fenretinide.

(A) Basal expression profile of twelve nuclear receptor genes in Huh-7, HepG2, and Hep3B cells. (B) Fenretinide induced nuclear receptors RARα, β, and γ mRNA in Huh-7 cells. (C) Fenretinide induced RARβ and Nor1 mRNA in HepG2 cells. (D) Comparison of RARβ mRNA level between Huh-7 and HepG2 cells after fenretinide treatment (relative fold of RARβ level between Huh-7 and HepG2 cells). Data from two independent experiments were presented as mean ± S.E.M.

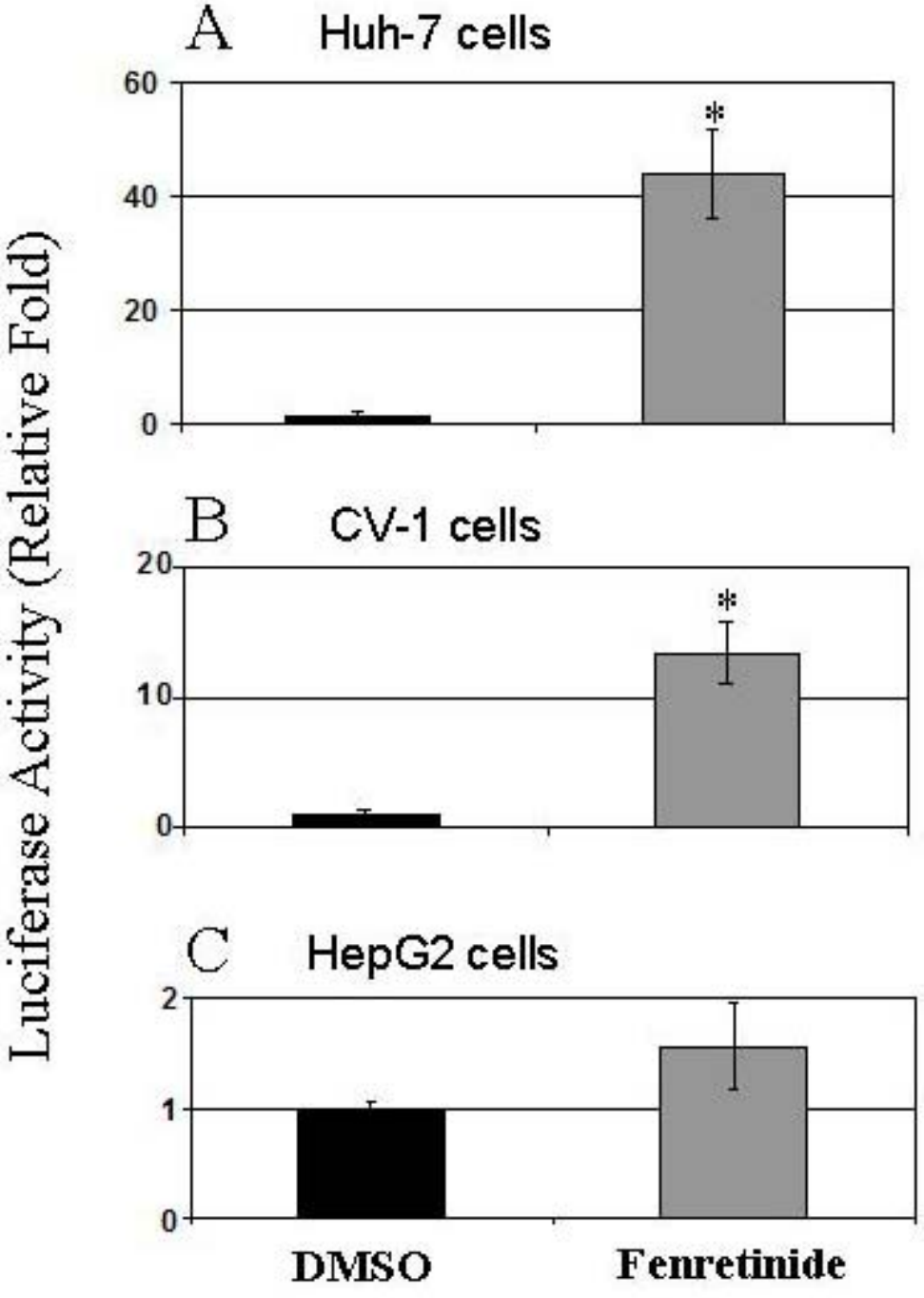
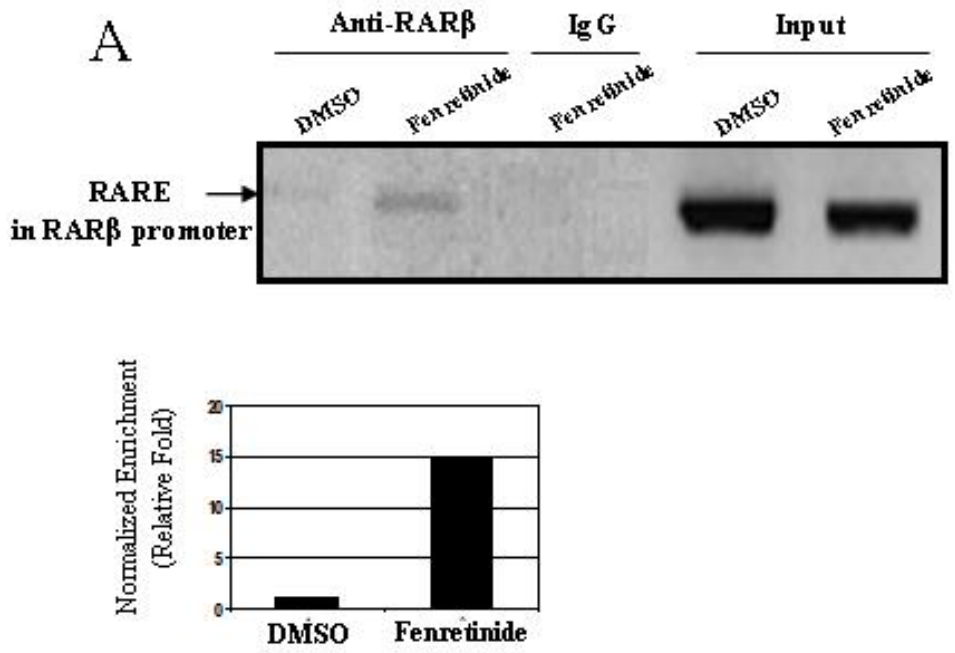

A
Huh-7 cells
60
40
20
0
*
B
CV-1 cells
20
10
0
*
C
HepG2 cells
2
1
0
DMSO
Fenretinide
Luciferase Activity (Relative Fold)

Figure 3.5: Fenretinide transactivates RXRα/RARβ-mediated pathway in Huh-7, CV-1 cells, but not in HepG2 cells.

Huh-7 (A), CV-1 (B), and HepG2 (C) cells were transfected with a luciferase reporter harboring RARE (DR-5) and expression plasmids for RXRα and RARβ. Firefly luciferase activity was normalized by co-transfected Renilla luciferase activity. The histograms depict the relative fold of normalized luciferase reporter activity between fenretinide treatment and DMSO treatment. Data from triplicates were presented as mean ± S.E.M.


A
Anti-RARβ
IgG
Input
DMSO
Fenretinide
Fenretinide
DMSO
Fenretinide
RARE
in RARβ promoter
Normalized Enrichment
(Relative Fold)
20
15
10
5
0
DMSO
Fenretinide

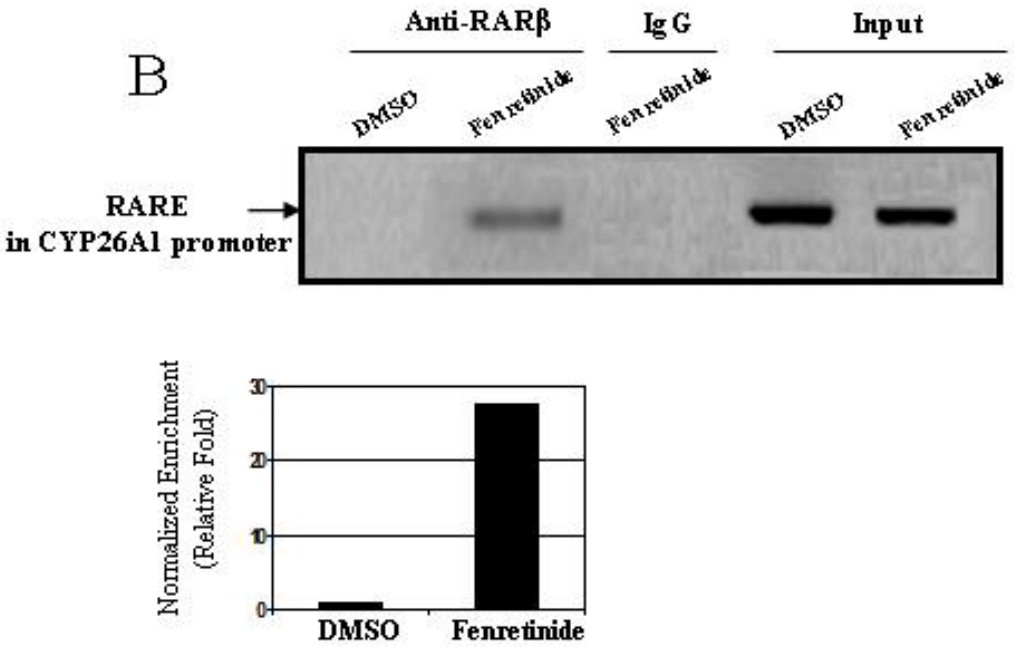
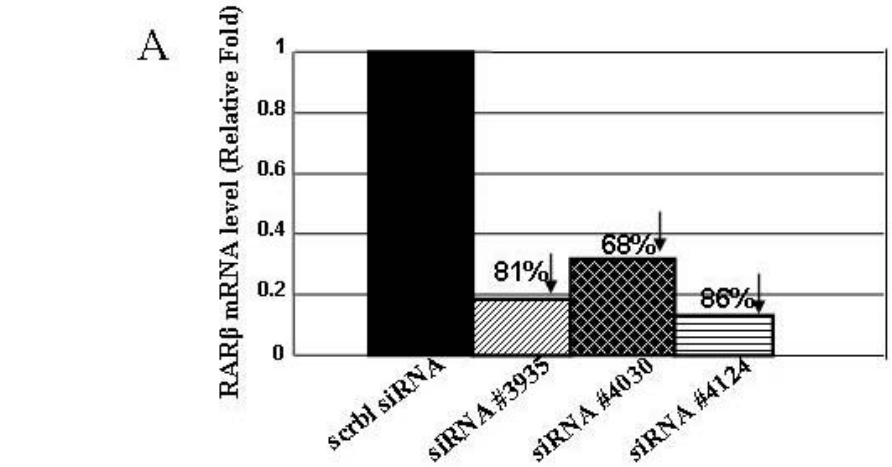

B
Anti-RARβ
IgG
Input
DMSO
Fenretinide
Fenretinide
DMSO
Fenretinide
RARE
in CYP26A1 promoter
Normalized Enrichment
(Relative Fold)
30
20
10
0
DMSO
Fenretinide

Figure 3.6: Fenretinide increases the transcriptional activity of RARβ.

Huh-7 cells were treated with fenretinide for 24 hours followed by ChIP assay. (A) RARβ and (B) CYP26A1 promoter fragments (harboring RARE) bound by RARβ were immunoprecipitated with a specific antibody against RARβ and amplified by PCR. Representative PCR results from two independent experiments were shown. The histograms depict the relative fold of PCR band intensity (after normalized to the corresponding input) between fenretinide treatment and DMSO treatment.


A
RARβ mRNA level (Relative Fold)
1
0.8
0.6
0.4
0.2
0
81%
68%
86%
scrbl siRNA
siRNA #3935
siRNA #4030
siRNA #4124

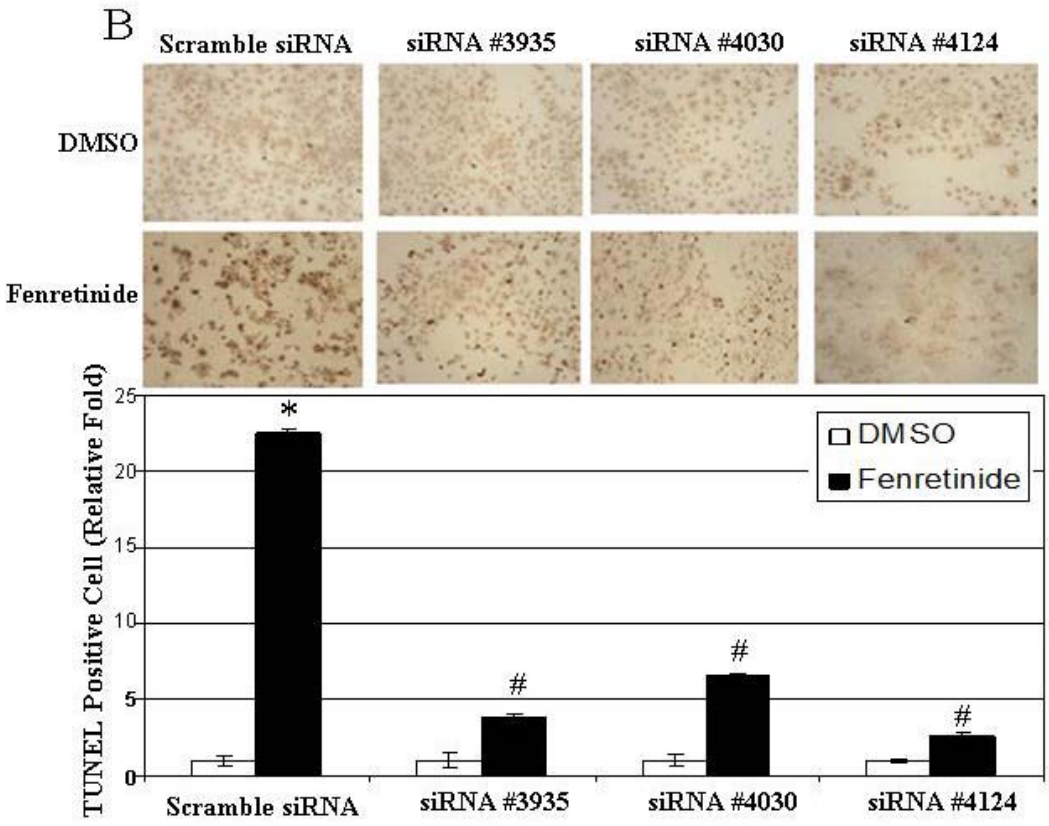

B
Scramble siRNA
siRNA #3935
siRNA #4030
siRNA #4124
DMSO
Fenretinide
TUNEL Positive Cell (Relative Fold)
25
20
15
10
5
0
*
#
#
#
DMSO
Fenretinide
Scramble siRNA
siRNA #3935
siRNA #4030
siRNA #4124

Figure 3.7: RARβ knockdown by siRNA diminished fenretinide-induced apoptosis. (A) Establishment of RARβ-deficient Huh-7 cells. Silencing of RARβ was achieved by transfecting Huh-7 cells with individual pre-designed RARβ siRNAs (#3935, #4030, and #4124) or scramble siRNA as the negative control. The knockdown efficiency of RARβ (i.e. RARβ mRNA level reduction) by each siRNA was presented as the percentage of RARβ mRNA level in scramble siRNA-transfected cells 48 hours post-transfection. (B) Scramble or RARβ siRNA transfected Huh-7 cells were treated with either DMSO or fenretinide for 24 hours followed by TUNEL assay. TUNEL staining was representative result from two independent experiments. The histograms depict the relative fold of TUNEL positive cells between fenretinide and DMSO treatment in individual siRNA transfected Huh-7 cells. TUNEL positive cell counting was presented as mean ± S.E.M. from four independent countings (* p <0.05, compared with DMSO treatment of scramble siRNA-transfected Huh-7 cells; $^{\#}$ p <0.05, compared with fenretinide treatment of scramble siRNA-transfected Huh-7 cells).

Discussion

Retinoids have emerged as important signaling molecules in the regulation of cellular homeostasis. During the past decade, the knowledge on the mechanisms of retinoids action has been greatly expanded due to the discovery and characterization of retinoid receptors and the consensus RAREs in retinoid target genes (Evans, 2005). Retinoid receptors are ligand-dependent transcription factors that regulate expression of retinoid target genes upon activation (Bastien and Rochette-Egly, 2004). One retinoid receptor, RARβ, has been speculated as a tumor suppressor in several studies. Decreased RARβ expression was found in head and neck squamous cell carcinoma (Xu et al., 1994), premalignant oral lesions (Lotan, 1995), and esophageal squamous cell carcinoma (Xu et al., 2005). Suppression of RARβ causes resistance to retinoic acid-associated growth arrest in breast and prostate cancer cells (Barna et al., 2005) and in F9 teratocarcinoma cells (Faria et al., 1999). Induced RARβ expression sensitizes non-small cell lung cancer cells and colorectal cancer cells to the anticancer effects of retinoids (Teraishi et al., 2003). However, how RARβ exerts its role as a tumor suppressor is largely unknown.

In the present study, we identified fenretinide from a panel of retinoids and carotenoids as the most effective one in inducing apoptosis in HCC cells. We further identified RARβ as the key nuclear receptor in mediating the effect of fenretinide. Moreover, evidence from this study clearly demonstrates that fenretinide directly activates RARβ and that RARβ is required for fenretinide-induced apoptosis in Huh-7 cells. Thus, the novel finding of the current study is the identification of a positive

correlation between RARβ expression and the susceptibility of HCC cells to fenretinide. This finding suggests a role of RARβ in determining the sensitivity of HCC cells to certain chemotherapeutic agents, which may also hold true for other types of tumor cells.

In fenretinide-resistant HepG2 cells, not only the basal RARβ mRNA level was low, but also the induction of RARβ mRNA by fenretinide was modest and discontinuous. It was known that the promoter of the RARβ gene is frequently hypermethylated in acute myeloid leukemia and cholangiocarcinoma (Rethmeier et al., 2006; Yang et al., 2005). Using DNA methyltransferase inhibitor, the basal RARβ mRNA level in HepG2 cells did not increase (unpublished data) which suggests that promoter methylation might not account for the suppressed RARβ mRNA expression in HepG2 cells.

Similar to the expression pattern of RARβ mRNA in HCC cells, the basal expression of Nurr1 is much higher in Huh-7 than in HepG2 cells suggesting Nurr1 might also contribute to the observed differential susceptibility. The basal expression level of Nur77 in Huh-7 and HepG2 cells also correlates with the sensitivity of the cell line to fenretinide-induced apoptosis. Nur77 was shown to enhance RARE activity in transactivation assay (Wu et al., 1997). Furthermore, recently studies suggest that Nur77 translocates to mitochondria and interacts with Bcl-2 to promote apoptosis (Black et al., 2004; Cao et al., 2004). Therefore, the role of Nur77 in fenretinide-induced apoptosis warrants further investigation.

Another major difference regarding the nuclear receptor basal expression pattern is that HepG2 cells express higher basal levels of CAR and SXR mRNA than Huh-7 cells. It is known that activation of CAR or constitutive activation of SXR induces hepatomegaly in mice (Huang et al., 2005; Staudinger et al., 2001; Xie et al., 2000). Whether the high levels of CAR and SXR contribute to the resistance of HepG2 cells to fenretinide-induced apoptosis should be investigated.

Fenretinide seems to be a rather stable compound. Pharmacokinetics studies have shown that fenretinide has a much longer elimination half-life than all-trans retinoic acid and 13-cis retinoic acid (Le Doze et al., 2000). In another study, the tissue concentration of fenretinide and its main metabolite N-(4-methoxyphenyl) retinamide (4-MPR) were determined after a 3-day treatment by HPLC (Vratilova et al., 2004). The data showed that the concentration of fenretinide was 5-fold higher than that of 4-MPR in various mouse tissues including liver. So it is unlikely that the observed apoptotic effect is mediated through fenretinide metabolites. In addition, in the present study, we used cell culture, in which the metabolism rate might not be as efficient as in the liver. To avoid accumulation of the metabolites of fenretinide during treatment, fresh retinoids were provided every 24 hours. So it is highly likely that the observed apoptotic effect was caused by the parent compound rather than the metabolites of fenretinide.

During fenretinide treatment, the other two RARs, RARα and γ, were highly induced after 48 hours (25-fold and 17-fold, respectively) in Huh-7 cells implicating the involvement of these two RARs at the late stage of apoptosis in Huh-7 cells. As

some studies have shown, RARα and RARγ may be involved in apoptosis induction in immortalized keratinocytes and leukemia cells (Papoutsaki et al., 2004; Robert et al., 2006). In HepG2 cells, however, NOR1 (also known as NR4A3), was induced 6-fold after 48 hours. This induction may contribute to the resistance of HepG2 cells to fenretinide as NOR1 has been suggested to have pro-survival functions in some cell types (Wang et al., 2006).

Another novel finding is the direct activation of RARβ by fenretinide. It has been shown that fenretinide induces apoptosis in many types of cancer cells including neuroblastoma cells, breast, lung, head and neck, cervical and ovarian cancer cells (Kim et al., 2002; Sun et al., 1999a). However, the underlying mechanisms are poorly understood. Some studies suggest that the effects of fenretinide are mediated through reactive oxygen species (ROS) and caspase-3 (Suzuki et al., 1999), whereas other studies indicate the involvement of ceramide (Maurer et al., 1999) and the NF-κB pathway (Simile et al., 2005). Both retinoid receptor dependent and independent mechanisms have been proposed for fenretinide anticancer effects (Sun et al., 1999a). Our results obtained from transactivation assay and ChIP assay clearly demonstrate that fenretinide directly activates RARβ in Huh-7 cells. Knockdown of RARβ mRNA expression by siRNA provides a direct proof that RARβ is required for fenretinide-induced apoptosis. To the best of our knowledge, this is the first study to report that nuclear receptor RARβ mediates the apoptotic effect of fenretinide in HCC cells. Our findings strongly suggest a potential role of RARβ as a tumor suppressor by mediating the signals of certain chemotherapeutic agents. However, there are still

unbridged gaps between RARβ activation and apoptosis execution. Exploration of RARβ target genes will provide helpful insights into these molecular links. In conclusion, our findings reveal that endogenous expression of retinoids receptor RARβ gene determines the susceptibility of HCC cells to fenretinide-induced apoptosis. Our results further demonstrate fenretinide directly activates RARβ and induces RARβ-dependent apoptosis in Huh-7 cells. These findings suggest a novel role of RARβ as a tumor suppressor by mediating the signals of certain chemotherapeutic agents.

CHAPTER FOUR

Orphan Nuclear Receptor Nur77 is Essential for Fenretinide-induced Apoptosis of Huh-7 Hepatocellular Carcinoma

Abstract

The synthetic retinoid fenretinide is known to induce apoptosis in various cancer cells. We previously reported that fenretinide effectively induced apoptosis in human hepatocellular carcinoma (HCC) Huh-7 cells but not in HepG2 cells. The current study examines the mechanisms underlying the differential susceptibilities of HCC cells to fenretinide-induced apoptosis. Our results showed that the induction of Nur77 mRNA positively correlated with the sensitivity of HCC cells to fenretinide treatment. Moreover, subcellular distribution patterns of Nur77 protein in response to fenretinide were characterized and compared between Huh-7 and HepG2 cells. It appears that Nur77 mediated the apoptotic effect of fenretinide in sensitive Huh-7 cells via actively targeting mitochondria whereas it conferred HepG2 cells resistance by specifically accumulating in the nucleus. In addition, Nur77 knockdown by siRNA in Huh-7 cells demonstrated that the proapoptotic factor Nur77 was required for the fully execution of fenretinide-induced apoptosis. Therefore, our findings demonstrate that Nur77 exerts opposing effects in HCC cells in response to fenretinide, which is, at least in part, responsible for the observed differential susceptibilities of HCC cells.

Introduction

Retinoids, natural and synthetic derivatives of vitamin A, have profound effects on cellular homeostasis including growth, differentiation, and apoptosis (Bastien and Rochette-Egly, 2004; Blomhoff and Blomhoff, 2006). Clinically, retinoids have displayed therapeutic activities against a number of proliferative diseases. One of the most significant examples is retinoic acid (RA)-mediated differentiation therapy for acute promyelocytic leukemia (APL) (Farhana et al., 2005). The use of RA changed APL from a highly lethal to a curable disease (a rate of complete remission over 90%), thus provided a strong rational for retinoid-based therapy (Okuno et al., 2004). A number of experimental studies and clinical trials have demonstrated that retinoids can inhibit or reverse the carcinogenic process in certain organs, including premalignant and malignant lesions in the oral cavity, head and neck, breast, skin and liver (Okuno et al., 2004).

Retinoid signaling is mediated by nuclear retinoid receptors such as retinoic acid receptor (RAR) and retinoid X receptor (RXR), which are ligand-dependent transcription factors. The observed induction of differentiation and anti-proliferation effects of retinoids are exerted through retinoid receptors. In the case of RA-mediated differentiation therapy for APL, a chromosomal translocation results in formation of a fusion protein of RARα and promyelocyte leukemia protein (PML) (RARα-PML). This fusion protein subsequently blocks myeloid differentiation. RA treatment relieves the suppression along the myeloid differentiation pathway and thus the APL

cells are able to differentiate into terminal neutrophils (Clarke et al., 2004; Fontana and Rishi, 2002).

Fenretinide (N-(4-hydroxyphenyl) retinamide, also known as 4HPR) is a structure analogue of all-trans retinoic acid that was first synthesized by R. W. Johnson Pharmaceuticals in the late 1960's. Abundant laboratory and clinical studies have demonstrated that fenretinide may hold great potential in cancer chemoprevention and therapy. Fenretinide not only inhibits cell proliferation but also induces apoptosis in a variety of human cancer cell types derived from head and neck cancer, non-small cell lung cancer, melanoma, prostate, bladder, neuroblastoma, leukemia, and cervical carcinoma (Hail et al., 2006). Furthermore, fenretinide exerts activities against carcinogenesis of the breast, prostate, pancreas, and skin in animal models (Abou-Issa et al., 1995; Ohshima et al., 1985). In clinical trials, fenretinide slowed the progression of prostate cancer in men and protected against the development of ovarian cancer and a second breast malignancy in premenopausal women (Hail et al., 2006; Veronesi et al., 1999). Therefore, fenretinide offers great promise as a therapeutic agent in cancer treatment and prevention.

Nur77 (NR4A1, also known as TR3, NGFI-B) belongs to nuclear receptor superfamily NR4A subfamily. Nur77 is one of the orphan nuclear receptors with no physiological ligands indentified so far. Nur77 is highly expressed in various tissues including thymus, pituitary gland, adrenal gland, muscle, lung, thyroid, and liver (Li et al., 2006). As a transcription factor, Nur77 binds to NGFI-B response element

(NBRE) and Nur-responsive element (NurRE) as monomer and homodimer, respectively (Maxwell and Muscat, 2006). In addition, Nur77 also forms heterodimers with RXR to mediate 9-cis retinoic acid signaling (Mangelsdorf and Evans, 1995; Perlmann and Jansson, 1995). It is worth noting that Nur77 is unique among nuclear receptors in that it is also an immediate-early gene induced by a diversity of extracellular stimuli, ranging from survival to differentiation and apoptosis. Most importantly, a number of studies have indicated that Nur77 plays important roles in chemotherapeutic agent-induced apoptosis. But the role of Nur77 in fenretinide-induced apoptosis has not been explored.

In the present study, we provide direct evidence that Nur77 is involved in mediating the apoptotic effect of fenretinide in HCC cells. Furthermore, our findings establish the distinct modes of action of Nur77 between the sensitive and resistant cells in response to fenretinide. For the first time, we have reported that the orphan nuclear receptor Nur77 mediates the apoptotic effect of fenretinide in sensitive Huh-7 cells while the same nuclear receptor also contributes to the resistance to fenretinide in HepG2 cells.

Results

Fenretinide induced caspase-3 cleavage in Huh-7 cells. We have previously reported that the synthetic retinoid, fenretinide, induced apoptosis only in Huh-7 but not in HepG2 cells (Bu and Wan, 2007). By 30 hours of treatment, fenretinide effectively induced caspase-3 cleavage as revealed by immunofluorescence staining with a specific antibody for cleaved caspase-3 (Figure 4.1). The cleaved/activated caspase-3 was distributed in both cytosol and nucleus degrading cellular substrates, a hall mark indicating ongoing apoptosis.

Fenretinide differentially induced Nur77 mRNA between Huh-7 and HepG2 cells. To examine the molecular mechanisms responsible for the distinct susceptibilities of HCC cells to fenretinide, we have quantified the mRNA level of a panel of nuclear receptors in both cell lines before and after fenretinide treatment (Bu and Wan, 2007). We found that RARβ was differentially induced by fenretinide between these two cell lines and that its induction pattern positively correlated with the susceptibility of HCC cells. In addition, we also identified a similar induction pattern for Nur77, an orphan nuclear receptor, between Huh-7 and HepG2 cells (Figure 4.2). This finding suggests that Nur77 may also contribute to the observed differential sensitivities of HCC cells.

Fenretinide induced distinct subcellular localization patterns of Nur77 protein between Huh-7 and HepG2 cells. We next examined the subcellular localization of Nur77 protein in response to fenretinide in Huh-7 and HepG2 cells. In

the sensitive Huh-7 cells, consistent with the induction of Nur77 mRNA by fenretinide, Nur77 protein was enriched in the cytosol after 24 hours of treatment, while such an induction pattern was absent in the control treatment (Figure 4.3, upper two rows). In addition, the up-regulated Nur77 protein was accumulated mainly in the cytosol but not in the nucleus. Concomitant with the progress of apoptosis, when the majority of cells were undergoing robust cell death after 30 hours of treatment as revealed by caspase-3 cleavage (Figure 4.1), Nur77 protein was distributed across the cell probably due to equilibrious diffusion (Figure 4.3, lower two rows). Remarkably, a distinct subcellular localization pattern of Nur77 protein in response to fenretinide was observed in the resistant HepG2 cells. In HepG2 cells, Nur77 protein was re-distributed ubiquitously across the cell after 24 hours of treatment, while in the control treatment Nur77 seemed to be excluded from the nucleus (Figure 4.4, upper two rows). After 48 hours Nur77 protein translocated into nucleus only in fenretinide treatment but not in the control treatment (Figure 4.4, lower two rows). Taken together, the distinct subcellular localization patterns of Nur77 between the sensitive and resistant cells in response to fenretinide suggest a potential role of Nur77 in mediating the observed differential sensitivities.

Fenretinide induced mitochondrial enrichment of Nur77 protein in Huh-7 but not in HepG2 cells. To determine the specific subcellular localization of Nur77 protein in response to fenretinide, subcellular fractions were isolated at different time points during fenretinide treatment in both cell lines followed by western blotting for Nur77, PARP (nuclear marker), and Porin (mitochondrial marker). Consistent with

the immunofluorescence staining data (Figure 4.3), Nur77 displayed distinct subcellular localization patterns between Huh-7 and HepG2 cells. In the sensitive Huh-7 cells, the total Nur77 protein appeared to be induced by fenretinide in a time-dependent manner. Moreover, the up-regulated Nur77 protein specifically relocated to mitochondria not nucleus, reaching its highest abundance after 24 hours of treatment (Figure 4.5A). After 36 hours of treatment, consistent with equilibrious diffusion pattern of Nur77 in immunofluorescence staining after 30 hours (Figure 4.3, lower two rows), an evenly distributed Nur77 protein was detected between nuclear and mitochondrial fractions. The reduced level of Nur77 protein is probably due to the degradation of Nur77 during apoptosis. In HepG2 cells, the total Nur77 protein did not change as markedly as it did in Huh-7 cells. In addition, Nur77 appeared to translocate from the mitochondria to the nucleus in a time-dependent manner. In agreement with immunofluorescence staining (Figure 4.4, lower two rows), Nur77 reached its highest abundance in the nucleus of HepG2 cells after 48 hours of treatment (Figure 4.5B).

Knockdown of Nur77 by siRNA partially blocked fenretinide-induced DNA double-strand breaks in Huh-7 cells. In order to determine whether Nur77 is required for fenretinide-induced apoptosis of Huh-7 cells, the endogenous Nur77 was knocked down by siRNA followed by TUNEL assay. Sequence-specific siRNA silenced endogenous Nur77 in a dose-dependent manner (Figure 4.6A). Nur77 siRNA at 50 nM achieved a marked knockdown effect (83.5% knockdown compared to scramble siRNA). After 24 hours of fenretinide treatment, robust DNA double-strand

breaks as revealed by TUNEL staining were detected in the cells transfected with scramble siRNA. On the contrary, only mild DNA double-strand breaks were seen in the cells transfected with Nur77 siRNA (Figure 4.6B). Therefore, these results clearly demonstrate that Nur77 is required for the full execution of fenretinide-induced apoptosis in Huh-7 cells.

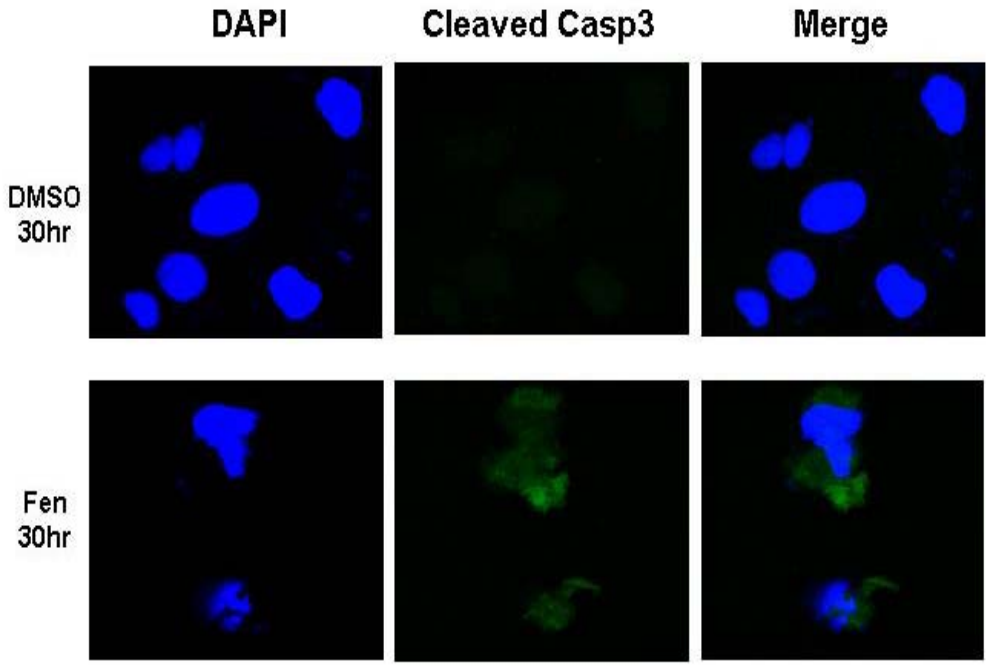
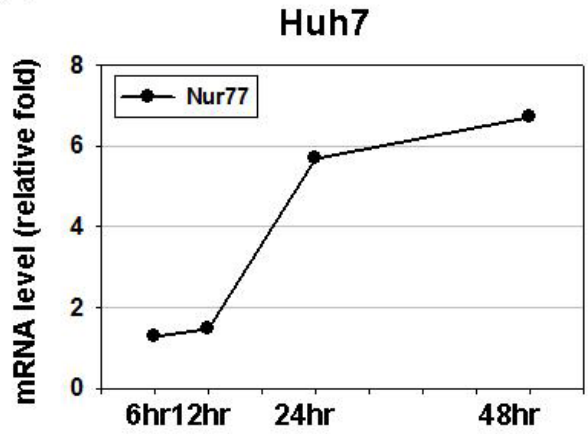


Figure 4.1: Fenretinide induced caspase-3 cleavage in Huh-7 cells. Huh-7 cells were incubated with either DMSO or fenretinide (10 μM) in serum-free medium for 30 hours followed by immunofluorescence staining with a specific antibody against cleaved caspase-3 as described in ***Materials and Methods***. Nuclei were counterstained with DAPI. Representative confocal microscopy images of duplicate treatments were shown.

A


Huh7
mRNA level (relative fold)
Nur77
8
6
4
2
0
6hr12hr
24hr
48hr

B

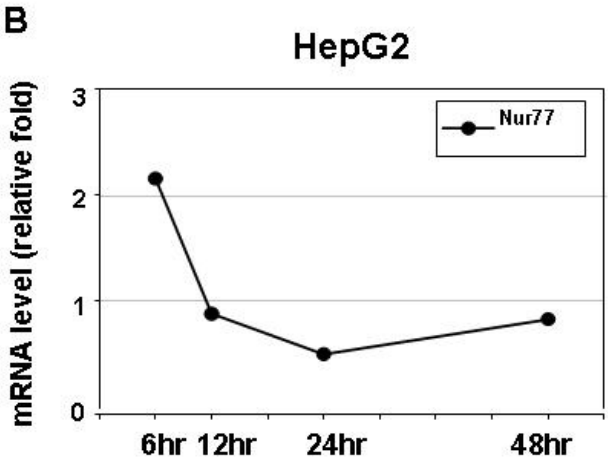

HepG2
mRNA level (relative fold)
Nur77
3
2
1
0
6hr 12hr
24hr
48hr

Figure 4.2: Fenretinide differentially induced Nur77 mRNA between Huh-7 and HepG2 cells. HCC cells were treated with either DMSO or fenretinide (10 μM) in serum-free medium for indicated time. Gene expression was analysed by quantitative real-time PCR using mRNA from (A) Huh-7 cells and (B) HepG2 cells with β-actin as the internal reference. The expression level of Nur77 was expressed as relative fold (fenretinide treatment vs. DMSO treatment) at each time point. Data were mean from duplicates.

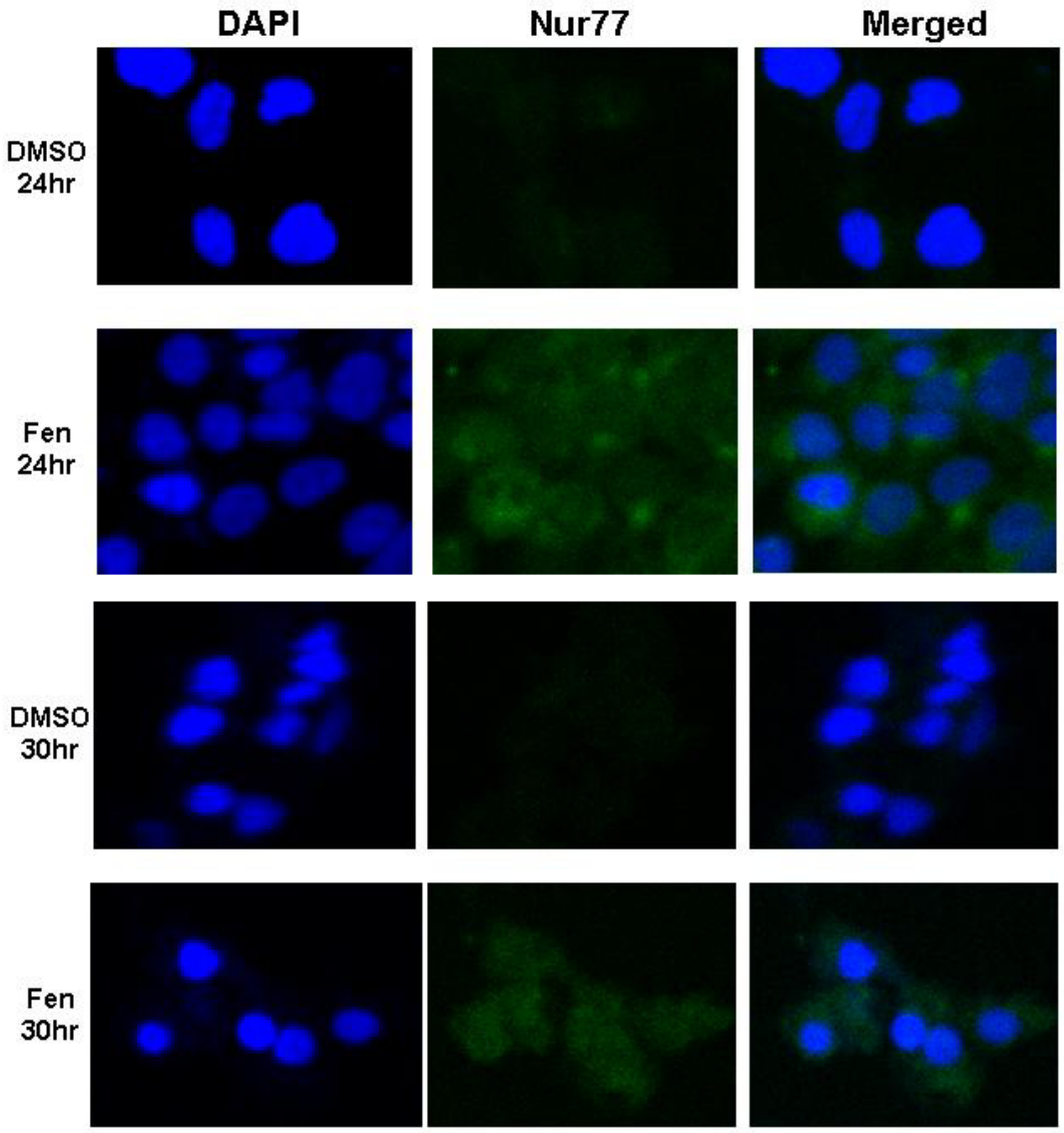

DAPI
Nur77
Merged
DMSO
24hr
Fen
24hr
DMSO
30hr
Fen
30hr

Figure 4.3: Fenretinide induced dynamic pattern of Nur77 protein subcellular localization in Huh-7 cells. Huh-7 cells were treated with either DMSO or fenretinide (10 μM) in serum-free medium for indicated time followed by immunofluorescence staining for Nur77. Nuclei were counterstained with DAPI. Representative confocal microscopy images were from two independent experiments.

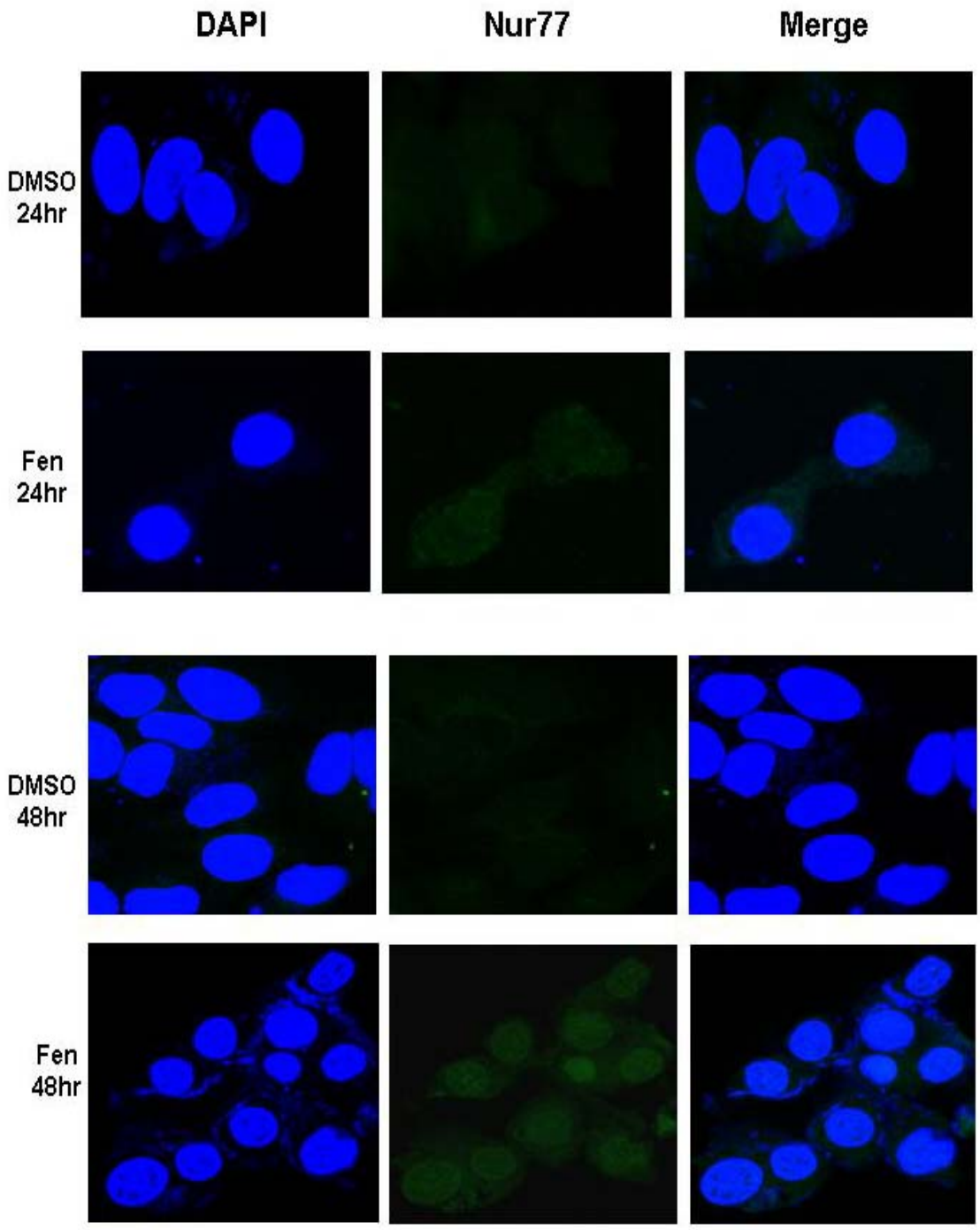
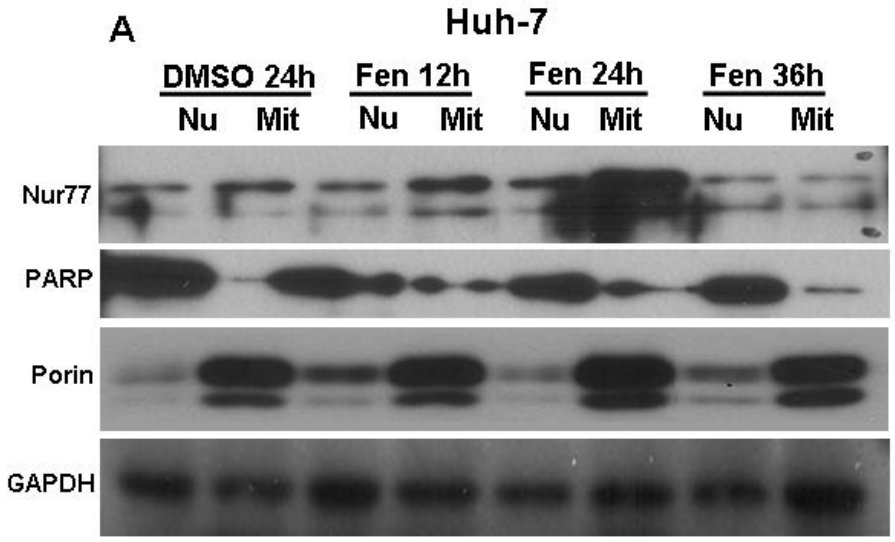

DAPI
Nur77
Merge
DMSO
24hr
Fen
24hr
DMSO
48hr
Fen
48hr

Figure 4.4: Fenretinide induced dynamic pattern of Nur77 protein subcellular localization in HepG2 cells. HepG2 cells were treated with either DMSO or fenretinide (10 μM) in serum-free medium for indicated time followed by immunofluorescence staining for Nur77. Nuclei were counterstained with DAPI. Representative confocal microscopy images were from two independent experiments.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Huh-7
DMSO 24h
Fen 12h
Fen 24h
Fen 36h
Nu
Mit
Nur77
PARP
Porin
GAPDH

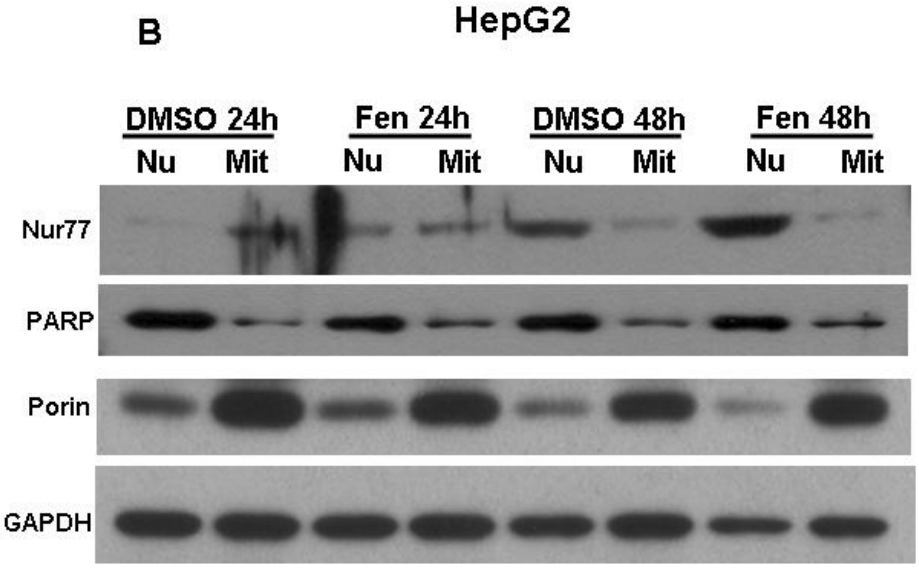
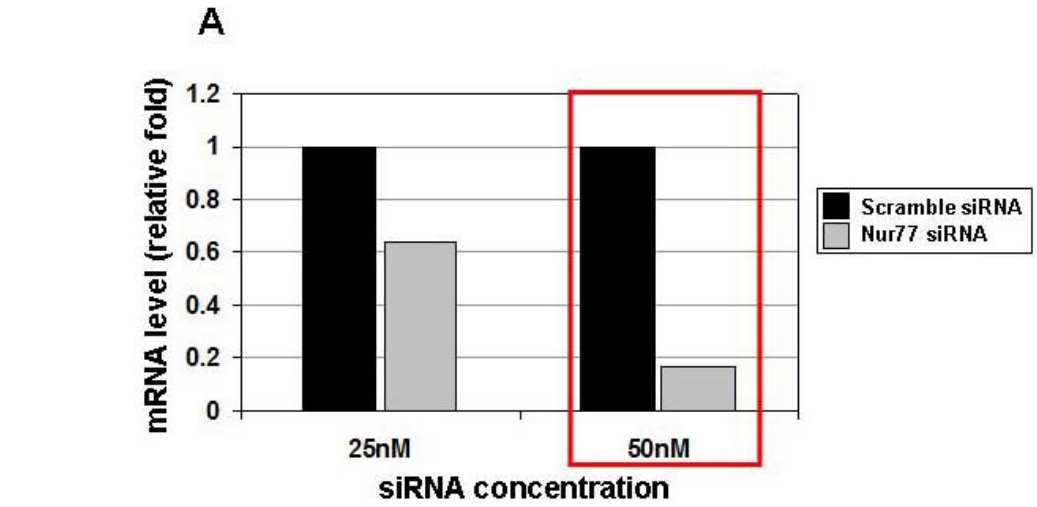

B
HepG2
DMSO 24h
Fen 24h
DMSO 48h
Fen 48h
Nu
Mit
Nur77
PARP
Porin
GAPDH

Figure 4.5: Fenretinide induced mitochondrial enrichment of Nur77 protein in Huh-7 cells but not in HepG2 cells. HCC cells were treated with either DMSO or fenretinide (10 μM) in serum-free medium for indicated time. Subcellular fractions were isolated as described in *Materials and methods* and stored at -20°C in aliquots until use. Protein levels of Nur77, PARP, and Porin from individual fractions of (A) Huh-7 and (B) HepG2 cells were analysed by western blotting. Membranes were incubated with antibodies specific to Nur77, PARP, and Porin. GAPDH was included as a loading control. Protein bands corresponding to target proteins (Nur77 p64, PARP p116, Porin 31, and GAPDH p40) are shown. Representative results were from three independent experiments.


A
mRNA level (relative fold)
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1
0.8
0.6
0.4
0.2
0
25nM
50nM
siRNA concentration
Scramble siRNA
Nur77 siRNA

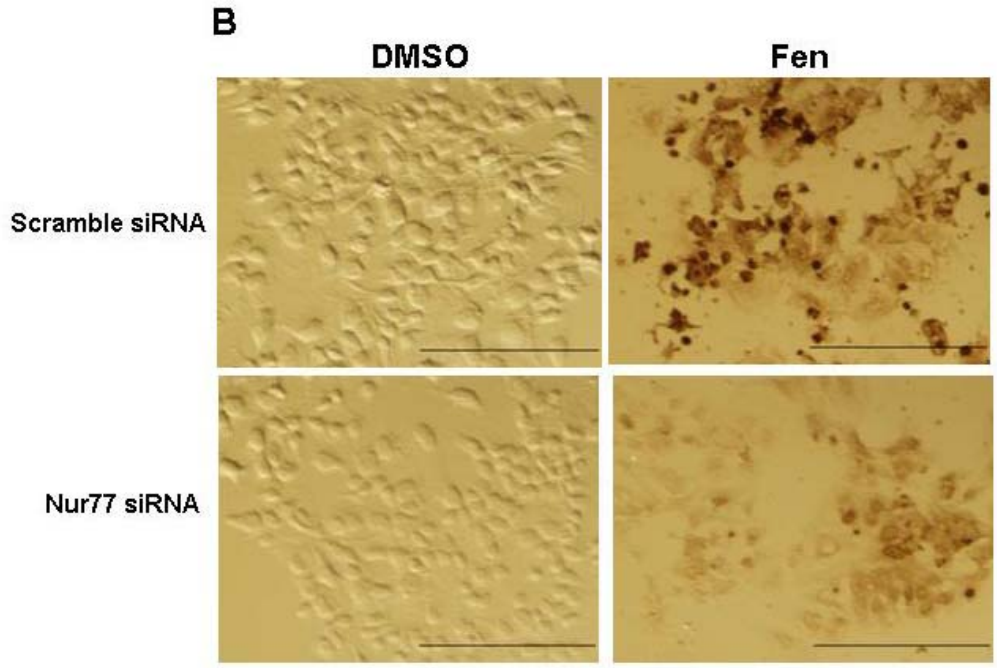

B
DMSO
Fen
Scramble siRNA
Nur77 siRNA

Figure 4.6: Knockdown of Nur77 by siRNA partially blocked fenretinide-induced DNA double-strand breaks in Huh-7 cells. Huh-7 cells were transfected with either scramble siRNA or Nur77 siRNA at concentrations of 25 and 50 nM for 48 hours. (A) Endogenous Nur77 mRNA level was analysed by quantitative real-time PCR using mRNA from each group and expressed as percentage relative to scramble siRNA group. (B) Scramble or Nur77 siRNA transfected Huh-7 cells were treated with either DMSO or fenretinide for 24 hours followed by TUNEL assay. Representative results of TUNEL staining were from duplicate experiments.

Discussion

Several mechanisms have been proposed for fenretinide-induced apoptosis of cancer cells. Intracellular signal molecules such as reactive oxygen species (ROS) and ceramide have been detected during fenretinide-induced apoptosis in a number of cell types (Hail et al., 2006). The most commonly observed property of fenretinide-induced apoptosis in cancer cells is its inhibition by antioxidants such as vitamin C, vitamin E, N-acetylcysteine, and pyrrolidine dithiocarbamate, thus suggesting an essential role for ROS and oxidative stress in fenretinide's cytotoxicity (Maurer et al., 1999; Oridate et al., 1997; Sun et al., 1999b). Similarly, fenretinide exposure also enhances cellular ceramide levels during apoptosis (Maurer et al., 1999). But inhibition of ceramide synthesis and metabolism provides inconsistent modulation of fenretinide apoptotic effect in various cancer cell types (Hail et al., 2006). Being a structure analogue of all-trans retinoic acid which has high affinity for retinoic acid receptor (RAR), fenretinide has been speculated to activate RAR. As expected, fenretinide was identified as a high affinity ligand for RAR and found to enhance RAR-mediated transcription in certain cell types including human hepatocellular carcinoma (HCC) cells and prostate carcinoma cells (Bu and Wan, 2007; Fanjul et al., 1996; Sun et al., 1999a). Moreover, our previous study has demonstrated that the susceptibilities of HCC cells to fenretinide positively correlate with their endogenous and inducible RARβ expression level, thus suggesting a role of RARβ in mediating the apoptotic effect of fenretinide (Bu and Wan, 2007). However, controversial evidence also exits. RAR knockout F9 embryonic carcinoma cells retained sensitivity

to apoptosis induction by fenretinide (Clifford et al., 1999). In neuroblastoma cells, RAR antagonists failed to suppress fenretinide-induced ROS generation (Lovat et al., 2000). Furthermore, neither all-trans retinoic acid nor 9-cis retinoic acid promoted ROS generation or apoptosis in neuroblastoma cells (Lovat et al., 2004), thus suggesting that fenretinide's cytotoxic effects were predominately independent of RAR activation in these cells. Therefore, the mechanisms responsible for fenretinide-induced apoptosis may be cell type-specific. It is also possible that fenretinide is capable of initiating multiple pathways in certain cellular settings.

In the present study, we have identified Nur77 as another mediator for fenretinide-induced apoptosis of HCC cells. We have found that not only the differential induction of Nur77 mRNA between Huh-7 and HepG2 cells but also its cellular distribution patterns positively correlated with susceptibilities of HCC cells to fenretinide treatment suggesting its important role in such a process. Remarkably, Nur77 has been reported to possess opposing biological functions in cell survival and death. Nur77 was originally identified as a serum and growth factor-inducible gene (Hazel et al., 1988; Lim et al., 1987). Nur77 is often found overexpressed in cancer cells including lung, prostate, breast, and colon cancer (Moll et al., 2006). In lung cancer cells, overexpression of Nur77 correlates with their resistance to retinoic acid-induced growth inhibition (Chen et al., 2002; Wu et al., 1997). Furthermore, both hypoxic conditions and vascular endothelial growth factor (VEGF) induce Nur77 expression, thus indicating that Nur77 may be a key mediator in angiogenesis (Liu et al., 2003; Yoo et al., 2004). On the contrary, a wealth of experimental data suggests

that Nur77 also mediates apoptosis signaling. Besides its documented roles in T cell receptor-mediated apoptosis, Nur77 was identified as the key mediator in apoptosis induced by the retinoid-related molecule, 6-(3-(1-adamantyl)-4-ydroxyphenyl)-2-naphtha-lenecarboxylic acid, also known as AHPN/CD437 (Li et al., 1998; Moll et al., 2006). Similarly, in the current study we have identified Nur77 as a mediator of apoptosis induced by a synthetic retinoid, fenretinide, in HCC cells. Furthermore, the death effect of Nur77 appears to be clinically relevant. Gene expression profiles of tumor samples from various cancer patients reveal an association between down regulation of Nur77 and metastasis of primary solid tumors including lung cancer, breast cancer, and prostate cancer (Ramaswamy et al., 2003). Therefore, a mechanistic understanding of the action of Nur77 in mediating apoptosis of cancer cells may have positive impact on chemotherapeutic applications.

Unlike most nuclear receptors which localize and function within the nucleus, Nur77 has exhibited an unusual mode of action when it mediates apoptosis signaling. In the absence of stimuli, Nur77 protein was found to predominantly reside in cytosol in many cell types (Davis et al., 1993; Fahrner et al., 1990; Hazel et al., 1991; Woronicz et al., 1995). Consistently, we found that a major portion of total Nur77 protein resided outside of nucleus in the control treatment (figure 3-5) in both Huh-7 and HepG2 cells. In the presence of diverse apoptotic stimuli, Nur77 migrates to mitochondria and induces cytochrome c release and subsequent apoptosis induction (Li et al., 2000). Importantly, it has been demonstrated that the active mitochondrial targeting function of Nur77, but not its DNA binding and transactivation activity, is

essential for its proapoptotic effect (Li et al., 2000; Wang et al., 2004). The direct supportive evidence is that the DNA-binding domain (DBD)-deleted Nur77 constitutively resides at mitochondria and causes extensive cytochrome c release (Li et al., 2000; Wang et al., 2004). Likewise, our results demonstrate that, in the sensitive Huh-7 cells, induced Nur77 protein specifically accumulated in mitochondrial fraction in a time-dependent manner in response to fenretinide (figure 5). Nur77 was reported to interact with an anti-apoptotic protein Bcl-2 at mitochondria and subsequently convert Bcl-2 to a proapoptotic molecule (Lin et al., 2004). Whether this is the case in fenretinide-induced apoptosis of Huh-7 cells warrants further examination. Interestingly, evidence was presented that Nur77 can also function as a survival factor when it accumulates in the nucleus (Moll et al., 2006; Zhang, 2007). Overexpression of Nur77 in H460 and Calu-6 lung cancer cells promotes DNA synthesis and cell cycle progression (Kolluri et al., 2003). Epidermal growth factor (EGF)-activated mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) phosphorylates Nur77 and leads to its predominant nuclear localization, which overcomes apoptosis-induced mitochondrial translocation and thus prohibits amplification of apoptosis (Jacobs et al., 2004; Slagsvold et al., 2002). In agreement, our results indicate that, in the resistant HepG2 cells, Nur77 accumulated within the nucleus time-dependently in response to fenretinide and thus conferred, at least in part, the cells resistance to fenretinide-induced apoptosis (figure 5).

In summary, the current study provides mechanistic evidence that may be responsible for the observed differential susceptibilities of HCC cells to apoptosis induced by the chemopreventive agent fenretinide. The cytosol-nucleus shuttling of orphan nuclear receptor Nur77 appears to be a key factor in such a determining process. Therefore, Nur77 may present a potential therapeutic target in cancer treatment as harnessing its subcellular localization may help manipulate the responsiveness of tumor cells to chemotherapy.

CHAPTER FIVE

13-cis Retinoic Acid Promotes Hepatocyte Proliferation via Activation of PPARβ Pathway

Abstract

Retinoids, derivatives of vitamin A, are known to induce differentiation but also to cause hepatomegaly in human and rodent. The current study examines the mechanism by which retinoids induce proliferation of hepatocellular carcinoma cells as well as in mouse livers. 13-cis retinoic acid (13-cis RA) stimulated proliferation of Hep3B cells, concomitant with elevated DNA synthesis and cell cycle progression. *In vivo*, 13-cis RA increased DNA synthesis in mouse livers after 5- and 10-day treatments. The high expression ratio of fatty acid binding protein 5 (FABP5) to cellular retinoic acid binding protein II (CRABP II) in Hep3B cells and in mouse livers prior to 13-cis RA treatment suggests that 13-cis RA may preferentially activate peroxisome proliferator activated receptor β (PPARβ) over retinoic acid receptor (RAR). After 13-cis RA treatment, the ratio of FABP5/CRABP II was further increased. Thus, 13-cis RA could activate PPARβ and induce the expression of PPARβ target genes including phosphoinositide-dependent protein kinase 1 (PDK-1) in Hep3B cells and mouse livers. Following induction of PDK-1, Akt was phosphorylated and activated in both Hep3B cells and mouse livers. Over-expression of PPARβ in Hep3B cells further enhanced 13-cis RA-induced DNA synthesis. Use of an Akt inhibitor blocked 13-cis RA-induced Akt activation and proliferation of Hep3B cells in a dose-dependent manner, but did not interfere with induction of PPARβ target genes indicating PPARβ is upstream of Akt. Activation of PPARβ/PDK-1/Akt cascade is thus responsible for the proliferative effect of 13-cis

RA. In conclusion, our findings demonstrate that 13-cis RA promotes hepatocyte proliferation through activation of PPARβ.

Introduction

Retinoids, derivatives of vitamin A, play important roles in differentiation, proliferation, and apoptosis. Clinically, retinoids are used to treat dermatological diseases such as acne and psoriasis and certain types of cancer including acute promyelocytic leukemia, cutaneous T cell lymphoma, and Kaposi's sarcoma (Fontana and Rishi, 2002).

Despite their important cellular functions, retinoids also have several adverse effects. Symptoms from hypervitaminosis A occur in multiple organs including skin, nervous system, musculo-skeletal system, circulation, and internal organs (Blomhoff and Blomhoff, 2006; Roenigk, 1989). Since vitamin A/retinoids are stored in the liver, hepatomegaly with or without abnormal liver function is frequently seen with vitamin A intoxication (Roenigk, 1989). Hepatomegaly may result from an increase in size of hepatocytes, or numbers of hepatocytes, or both. Retinoid-stimulated hepatocyte proliferation may be one of the key mechanisms responsible for vitamin A-induced hepatomegaly. Besides vitamin A, the proliferative effect of other retinoids has also been reported. All-trans retinoic acid (all-trans RA) stimulates proliferation of human T lymphocytes and mouse hepatocytes (Engedal et al., 2006; Ledda-Columbano et al., 2004; Ohmura et al., 1996). All-trans RA also facilitates tumor growth in a transgenic mammary cancer mouse model and exerts an anti-apoptotic effect in HaCaT and NaF cells (Schug et al., 2007). Another natural retinoid, 9-cis RA, has been demonstrated as a hepatocyte mitogen in rats (Ohmura et al., 1996). In a previous screening of a panel of retinoids on human hepatocellular carcinoma cells, we found that 9-cis

retinaldehyde, 13-cis retinaldehyde, and 13-cis RA could stimulate the proliferation of Hep3B human hepatocellular carcinoma cells in the absence of serum (Bu and Wan, 2007). Thus, certain retinoids possess proliferative effect in addition to their well-known differentiation effect. The proliferative effect of retinoids may be retinoid-specific and/or cellular context-dependent although the underlying mechanisms remain largely unknown.

Retinoid binding proteins have an important role in intracellular stabilization of retinoids. During their transport in the aqueous cytosol, the hydrophobic-natured retinoids are solubilized and stabilized by binding to these binding proteins. Recent studies indicate that these binding proteins also participate in mediating the biological activities of retinoids (Dong et al., 1999; Tan et al., 2002). Cellular retinoic acid binding protein (CRABP) I and II possess a high affinity for retinoic acid (RA). CRABP I can decrease the cellular responses to RA by promoting its degradation, while CRABP II can deliver RA to retinoic acid receptor (RAR) and thus facilitate RAR-mediated signaling (Budhu and Noy, 2002; Dong et al., 1999). Both CRABP I and II belong to a binding protein superfamily which also includes fatty-acid binding proteins (FABPs) (Chmurzynska, 2006). FABP 5 (also known as keratinocyte FABP) and FABP 4 (also known as adipocyte FABP) have been shown to enhance the transcriptional activity of peroxisome proliferator-activated receptor (PPAR) β and γ, respectively (Tan et al., 2002). Upon ligand binding, these FABPs translocate to the nucleus and selectively activate PPAR subtypes (Schug et al., 2007; Tan et al., 2002).

Therefore, FABPs may be essential in regulating PPAR-mediated signaling pathways by selectively delivering the ligands to specific PPARs.

PPARβ (also known as δ) together with PPARα and PPARγ belongs to the nuclear hormone receptor superfamily PPAR (NR1C) subfamily (1999; Germain et al., 2006c). PPARβ is ubiquitously expressed in the body (Kliewer et al., 2001). A recent study of the quantitative expression pattern of PPARβ in mice revealed that the expression of PPARβ is highest in colon, small intestine, liver, and keratinocytes as compared to other tissues, suggesting a significant biological function of this nuclear receptor in these tissues (Borland et al., 2008). There is controversial evidence in the literature regarding the role of PPARβ in growth and differentiation of epithelial cells (Burdick et al., 2006). PPARβ displays a survival role in keratinocyte homeostasis during inflammation and wound healing (Di-Poi et al., 2003; Di-Poi et al., 2002; Icre et al., 2006). However, other studies show that ligand activation of PPARβ induces terminal differentiation of neoplastic keratinocytes and inhibits chemically induced skin tumorigenesis in mice (Bility et al., 2008). PPARβ agonists also inhibit growth of HaCaT and N/TERT-1 human keratinocyte cell lines (Borland et al., 2008; Burdick et al., 2007). Activation of PPARβ stimulates proliferation of human breast cancer and prostate cancer cells (Stephen et al., 2004). On the contrary, ligand activation of PPARβ fails to significantly modulate cell proliferation in A549 and H1838 human lung cancer cell lines (He et al., 2008). Therefore, the proliferative effect of PPARβ seems to be tissue/cell type specific.

In the present study, we studied the mechanism underlying 13-cis RA-induced proliferation of Hep3B cells and mouse livers. Our results establish that in liver cells, 13-cis RA activates a signaling cascade that initiates from activation of PPARβ and ends with activation of Akt. For the first time, we have identified a mechanism in the liver that is responsible for the long observed retinoid-induced hepatomegaly.

Results

13-cis RA stimulated proliferation of Hep3B cells. By screening a panel of retinoids, we have reported that 9-cis retinaldehyde, 13-cis retinaldehyde, and 13-cis RA stimulated proliferation of Hep3B cells (Bu and Wan, 2007). The proliferative effect of 13-cis RA was relatively stronger than that of the other two retinoids. To further confirm this finding, Hep3B cells were treated with 13-cis RA for 3 days and cell viability was monitored every 24 hrs. 13-cis RA treatment increased cell number in a time-dependent manner as assessed by cell counting and DNA synthesis/BrdU incorporation assay (Figure 5.1A and B). Thus, 13-cis RA is a mitogen for Hep3B cells.

13-cis RA enhanced cell cycle progression of Hep3B cells. Since cellular proliferation is associated with cell cycle progression, protein levels of several key cell cycle regulators were assessed after 13-cis RA treatment. The protein levels of cyclin D1 and D3 were increased after 2 days of treatment. The level of cyclin-dependent kinase 6 (CDK-6) was continuously increased after 2 and 4 days, whereas the level of CDK-4 remained unchanged. In contrast, the levels of cell cycle inhibitors, such as p21 and p27, were reduced by 13-cis RA (Figure 5.2). These results suggest that 13-cis RA facilitates the transition of Hep3B cells through the G1/S check point and thereby promotes cell cycle progression.

13-cis RA induced hepatocyte proliferation in mice. In addition to *in vitro* proliferative effects, 13-cis RA administered by gastric gavage also exerted a mitogenic effect in the mouse liver. 13-cis RA induced DNA synthesis in mouse

livers after 5 and 10 days of treatment when compared to the vehicle control groups. Moreover, 13-cis RA-induced DNA synthesis in non-parenchymal cells was even more prominent by 10 days (Figure 5.3A and B). In addition, the liver-to body weight ratio was increased by 7% after 10-day 13-cis RA treatment (data not shown).

High FABP5/CRABP II ratio before and after 13-cis RA treatment may favor the selective activation of nuclear receptor PPARβ. Treatment with all-trans RA facilitates tumor growth in a transgenic mammary cancer mouse model and protects HaCaT human keratinocyte cells from TNFα-induced apoptosis (Schug et al., 2007). In both cases, a high ratio of FABP5/CRABP II exists prior to treatment. Moreover, decreasing the FABP5/CRABP II ratio in HaCaT or NaF Cells converts RA from a survival to a pro-apoptotic factor (Schug et al., 2007). Thus, we examined the expression ratio of FABP5 and CRABP II in Hep3B cells and mouse livers before and after 13-cis RA treatment. The basal mRNA level of FABP5 was much higher than that of CRABP II in both Hep3B cells (39-fold) and mouse livers (213-fold) (Figure 5.4). After 13-cis RA treatment, the ratio of FABP5/CRABP II was even greater (Figure 5.4). The mRNA level of each gene at denoted time points is expressed as fold relative to that of CRABP II at day 0 for both Hep3B cells and mouse livers. In Hep3B cells, 13-cis RA decreased the expression of both CRABP II and FABP5, but the reduction of CRABP II was much greater than that of FABP5 (Figure 5.4A). In mouse livers, 13-cis RA induced the mRNA level of both CRABP II and FABP5, but the induction of FABP5 was much greater than that of CRABP II (Figure 5.4B). Therefore, the overall FABP5/CRABP II ratio was further increased in

both Hep3B cells and mouse liver in response to 13-cis RA. The cellular binding protein FABP5 plays an essential role in transporting RA to PPARβ (Schug et al., 2007). RA also has a high binding affinity to PPARβ (Shaw et al., 2003). Therefore, the high ratio of FABP5/CRABP II in Hep3B cells and mouse livers suggests a selective activation of PPARβ by 13-cis RA.

13-cis RA activated PPARβ as an effective ligand in both Hep3B cells and mouse livers. PPARβ dimerizes with RXR and binds to the cognate response elements (PPRE) residing in the promoter region of their target genes in response to ligand stimulation (Kliewer et al., 2001). 13-cis RA markedly induced the PPRE-reporter gene when RXRα and PPARβ were co-expressed (Figure 5.5A). In contrast, a weak induction was observed with RXRα alone, and no induction was detected with PPARβ alone (Figure 5.5A). In Hep3B cells, 13-cis RA consistently induced PPARβ target genes, adipose differentiation-related protein (ADFP) and 3-phosphoinositide dependent protein kinase-1 (PDK-1) throughout the 4-day treatment (Figure 5.5B) (Chawla et al., 2003; Di-Poi et al., 2002). In mouse livers, a marked induction of PPARβ target genes, mPDK-1 (9.9 fold) and mFABP3 (6.2 fold) was detected after a 10-day treatment with 13-cis RA (Figure 5.5C) (Di-Poi et al., 2002; Holst et al., 2003). Therefore, the present data demonstrate that 13-cis RA activates the PPARβ-mediated pathways in Hep3B cells and mouse livers.

13-cis RA activated PPARβ/PDK-1/Akt pathway in Hep3B cells and mouse livers. PDK-1, a PPARβ target gene, was induced by 13-cis RA in both Hep3B cells and mouse livers. PDK-1 is an important component of the anti-

apoptotic/proliferative PPARβ pathway where induction of PDK-1 leads to activation of the downstream survival factor Akt (Di-Poi et al., 2002). Thus, the effect of 13-cis RA on the protein level of PDK-1 and phosphorylation of Akt was examined. 13-cis RA markedly induced PDK-1 protein and increased Akt phosphorylation at Thr308, a specific phosphorylation site by PDK-1 and also a molecular marker for Akt activation (Figure 5.6A). Similarly, 13-cis RA induced the protein level of mPDK-1 and increased phosphorylation of Akt at Thr308 in mouse livers throughout the 10-day treatment (Figure 5.6B). Hence, 13-cis RA-induced proliferation in Hep3B cells and mouse livers might be mediated through the PPARβ/PDK-1/Akt pathway.

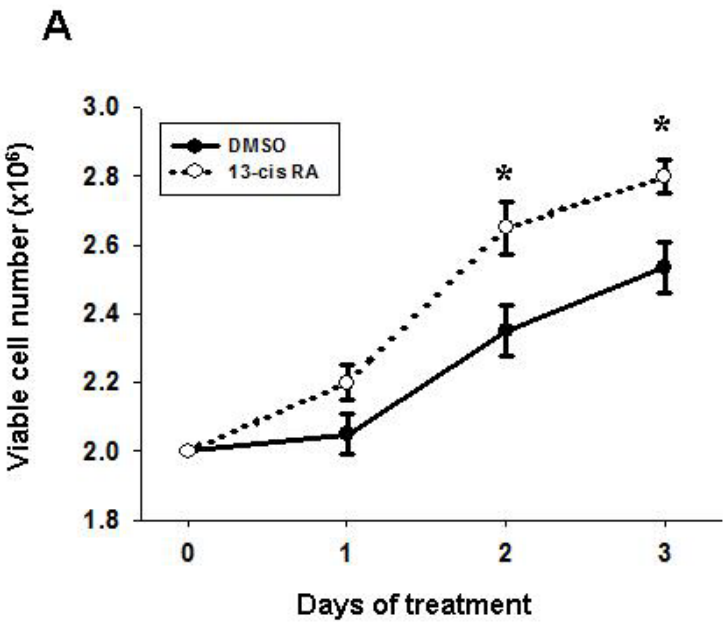
Over-expression of RXRα and PPARβ further stimulated 13-cis RA-induced proliferation of Hep3B cells. To establish the role of PPARβ and RXRα in mediating 13-cis RA-induced proliferation, PPARβ and RXRα were ectopically expressed in Hep3B cells by transfection. The elevated PPARβ protein was confirmed by western blotting (Figure 5.7A). Overexpression of PPARβ and RXRα further enhanced 13-cis RA-induced DNA synthesis when compared to vector transfection (Figure 5.7B). These results clearly demonstrate that 13-cis RA-induced proliferation of Hep3B cells is mediated through the PPARβ pathway.

Akt inhibitor blocked 13-cis RA-induced Akt activation and proliferation, and lead to a compensatory induction of the PPARβ/PDK-1/Akt pathway. To determine whether Akt activation is essential for 13-cis RA-induced proliferation, the pharmacological Akt inhibitor (1,3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one

trifluoroacetate salt hydrate) was used. This inhibitor is reported to be potent and selective to Akt isozymes, with an IC_{50} of 58 nM, 210 nM, and 2.12 mM for Akt1, Akt2, and Akt3, respectively (Zhao et al., 2005). The inhibition of Akt by this inhibitor was confirmed by western blotting with a specific antibody for phospho-Akt at Ser473, which is the marker of Akt activation. 13-cis RA-induced proliferation of Hep3B cells was blocked by Akt inhibitor in a dose-dependent manner (Figure 5.8A) that correlated with the decreased Akt phosphorylation level in the treated cells (Figure 5.8B). This inhibitor interfered with Akt phosphorylation that occurred downstream of PDK-1, and did not affect PDK-1 protein induction by 13-cis RA (Figure 8B). Akt inhibitor and 13-cis RA combination further caused a compensatory induction in the mRNA levels of PPARβ and PDK-1 compared to 13-cis RA alone (Figure 8C). Therefore, these data indicate that Akt activation is essential for 13-cis RA induced proliferation of Hep3B cells.

Table 5.1: Real-time PCR primers and probes used in this study.

Target Gene	Gene Bank Acession NO.		Primer Sequence (5'-3') Sequences are shown for forward (F) and reverse (R) primers	Probe Sequence (5'-3') with modification of 5' FAM / 3' BHQ1
hCRABP II	NM_001878.2	F	CCACCCCCTCCTTCTAGGATA	CGCTCCCCTTACCCCAGTCACTTCTG
		R	GCAAGAGGCATCCCAGTGA	
hFABP5	NM_001444.1	F	CCCTGGGAGAGAAGTTTGAAGA	ACCACAGCTGATGGCAGAAAAACTCAG AC
		R	AATGCACCATCTGTAAAGTTGCA	
hPDK-1	NM_002613	F	GAGATTGTGTCTGCTTTAGAGTACT TG	TCACAGGGACCTTAAACCGGAAAACAT TTT
		R	CTGTGATCTGGATGTGCATATCTTC	
hADFP	NM_001122	F	CCTGCTCTTCGCCTTTCG	TGCAGTCCGTCGATTTCTTTCTCCAGG
		R	TGCAACGGATGCCATTTTT	
hPPARβ/δ	NM_006238.3	F	TACAATGCCTACCTGAAAAACTTCA	CATGACCAAAAAGAAGGCCCGCAG
		R	GGCTTTGCCGGTGAGGAT	
hβ-Actin	NM_001101	F	CCTGGCACCCAGCACAAT	ATCAAGATCATTGCTCCTCCTGAGCGC
		R	GCCGATCCACACGGAGTACT	
mCrabp II	NM_007759	F	CCAGGGTCTACGTCCGAGAGT	TGCCTACGGGTCCAAGAACTGCCTG
		R	TGTAGCGGGCACGGAAGT	
mFabp5	NM_010634	F	AGATGATCGTGGAGTGTGTCATG	ACAATGCCACCTGCACTCGGGTCT
		R	GCCCTCATTGCACCTTCTCA	
mPdk-1	NM_011062	F	CGCCCCTCTTAGCATCCA	ACTGCATGCCTGCCTCTACCTTTCCC
		R	ACCCCGTCCCTCAGTCACA	
mFabp3	NM_010174	F	CCCCTCAGCTCAGCACCAT	CCTCATGGTTTTCCCCTCTGACATTTTGT
		R	GAAAAATCCCAACCCAAGAATG	
mβ-Actin	NM 007393	F	CTT CTT TGC AGC TCC TTC GTT G	CCA CAC CCG CCA CCA GTT CGC C
		R	CGA CCA GCG CAG CGA TAT C	


A
3.0
2.8
2.6
2.4
2.2
2.0
1.8
Viable cell number (x10^6)
DMSO
13-cis RA
*
*
0
1
2
3
Days of treatment

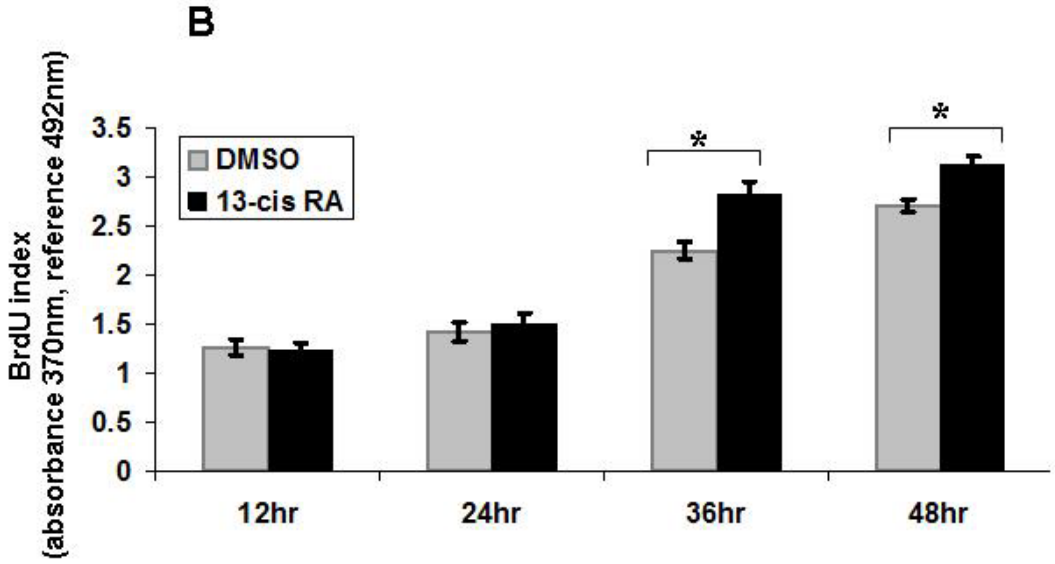

B
BrdU index
(absorbance 370nm, reference 492nm)
3.5
3
2.5
2
1.5
1
0.5
0
DMSO
13-cis RA
*
*
12hr
24hr
36hr
48hr

Figure 5.1: 13-cis RA stimulated proliferation of Hep3B cells. Hep3B cells were incubated with either DMSO or 13-cis RA (10μM) in serum-free medium for indicated time. (A) Viable cell number was determined by trypan blue exclusion counting as described in ***Materials and Methods***. (B) BrdU incorporation index was measured as described in ***Materials and Methods***. Normalized absorbance was expressed as mean ± S.E. from four replicates (* $p < 0.05$ compared to DMSO treatment). Representative results of two independent experiments are shown.

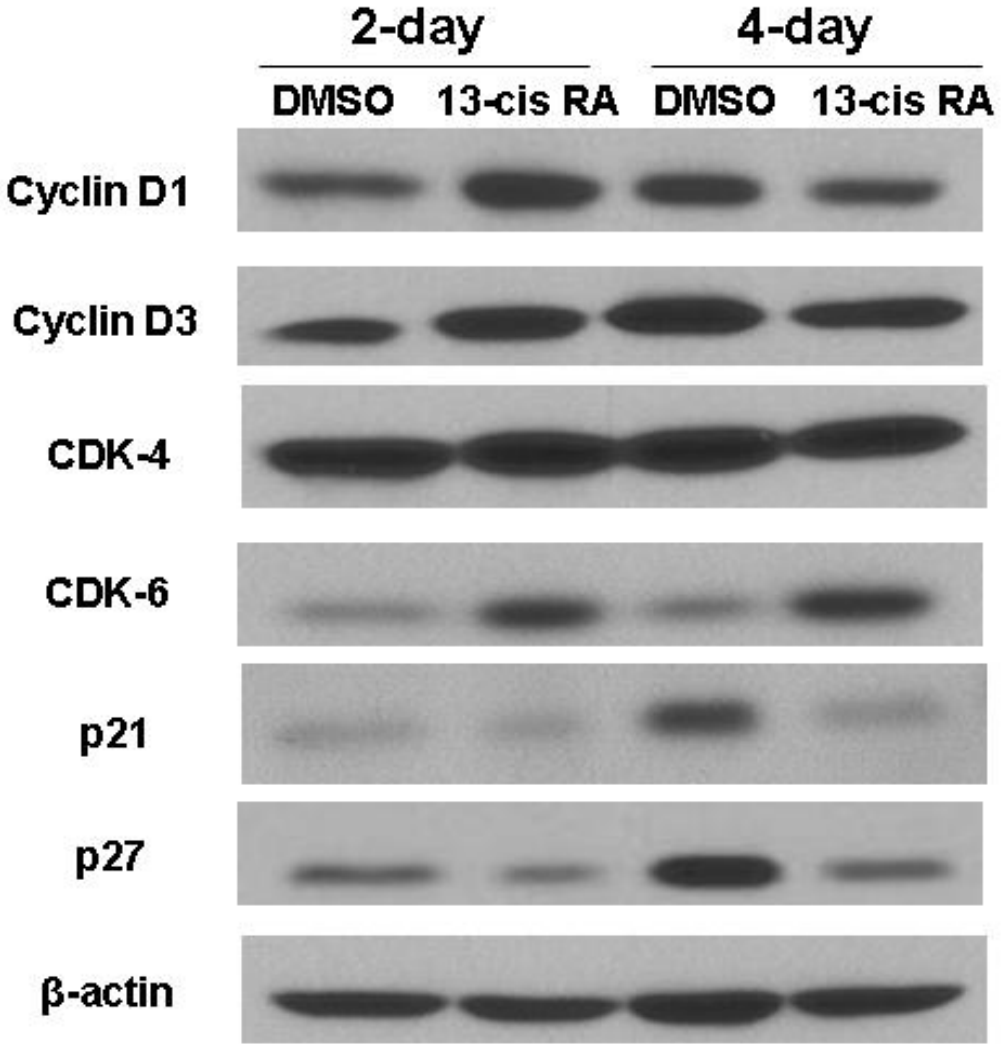

2-day
4-day
DMSO
13-cis RA
DMSO
13-cis RA
Cyclin D1
Cyclin D3
CDK-4
CDK-6
p21
p27
β-actin

Figure 5.2: 13-cis RA enhanced cell cycle progression of Hep3B cells. Protein levels of some key cell cycle regulators in the whole cell extracts of Hep3B cells treated with or without 13-cis RA at indicated time points were analysed by western blotting. Membranes were incubated with an antibody specific to each cell cycle regulator: Cyclin D1, Cyclin D3, Cyclin-dependent kinase 4 and 6 (CDK-4 and -6), p21, and p27. β-Actin was included as a loading control.

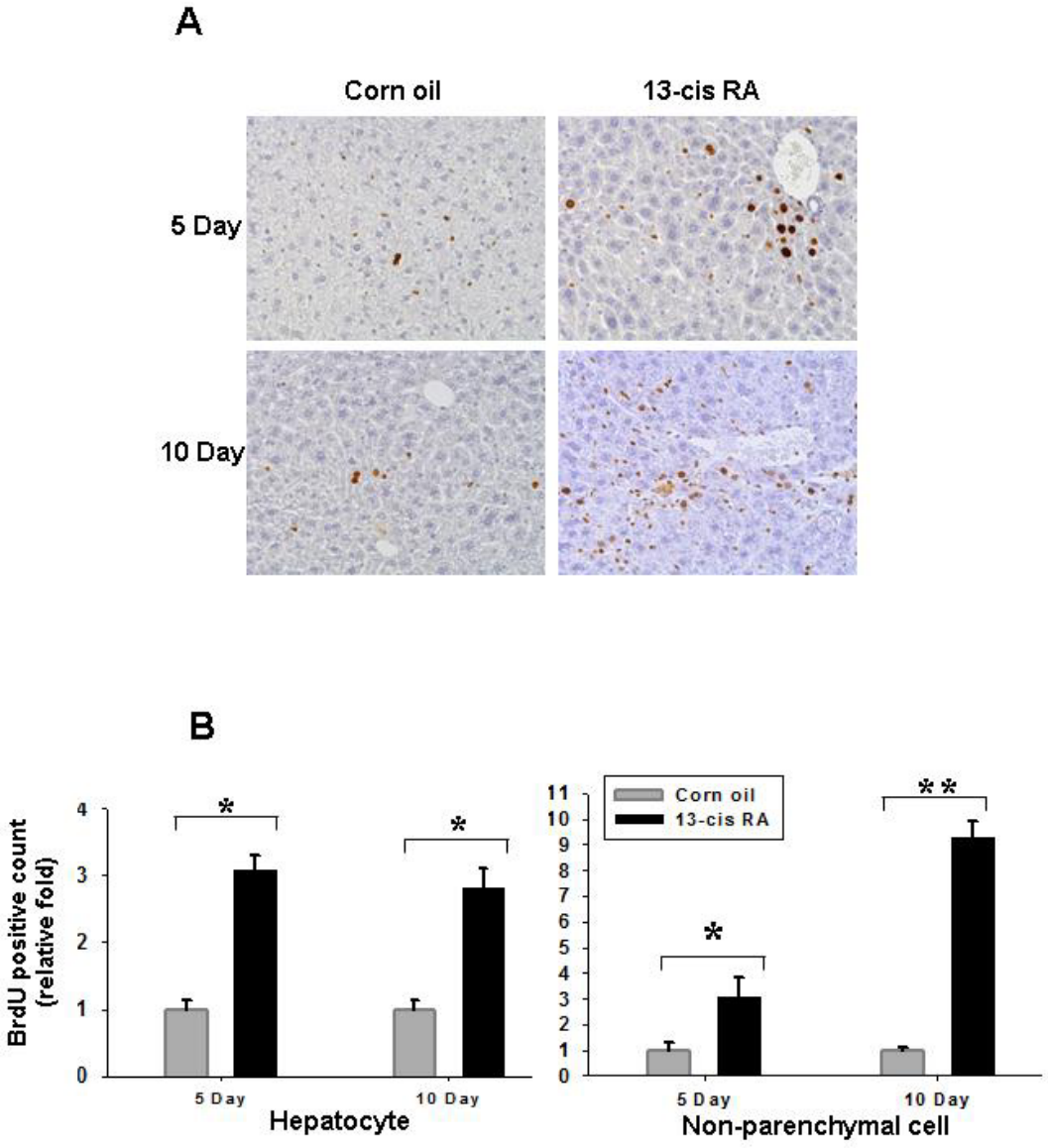

A
Corn oil
13-cis RA
5 Day
10 Day
B
BrdU positive count (relative fold)
0
1
2
3
4
*
*
5 Day
10 Day
Hepatocyte
Corn oil
13-cis RA
0
1
2
3
4
5
6
7
8
9
10
11
*
**
5 Day
10 Day
Non-parenchymal cell

Figure 5.3: 13-cis RA stimulated cellular proliferation in mouse livers. Mice (n=4-5) were gavaged with either corn oil or 13-cis RA for the indicated time. (A) Representative immunohistochemical staining of BrdU incorporation in mouse livers is shown. (B) Quantification of BrdU positive hepatocytes and non-parenchymal cells in mouse livers. Data from four microscopic fields were presented as fold relative to corn oil treatment $\pm$ S.E. (* $p < 0.05$, ** $p<0.01$, compared to corn oil treatment).

A

Hep3B	Day 0	Day 2	Day 4	Day 6
CRABP II	1	0.037	0.049	0.061
FABP5	39.12	3.56	3.45	5.98
ratio	39.12	95.67	70.28	97.34

B

Mouse liver	Day 0	Day 1	Day 2
mCRABP II	1	68.4	48.5
mFABP5	213.04	34278.1	28329.2
ratio	213.04	501.46	584.07

Figure 5.4: High FABP5/CRABP II ratio before and after 13-cis RA treatment may favor the selective activation of nuclear receptor PPARβ. Gene expression was analysed by quantitative real-time PCR using mRNA from (A) Hep3B cells before and after 13-cis RA treatment and (B) pooled mRNA from livers of mice (n=4-5) before and after 13-cis RA gavage with β-actin as the internal reference. The expression level of each gene at denoted time points was expressed as fold relative to that of CRABP II at day 0 in (A) Hep3B cells and (B) mouse livers. Data were presented as mean from duplicates. Representative data from two real-time PCR experiments are shown.

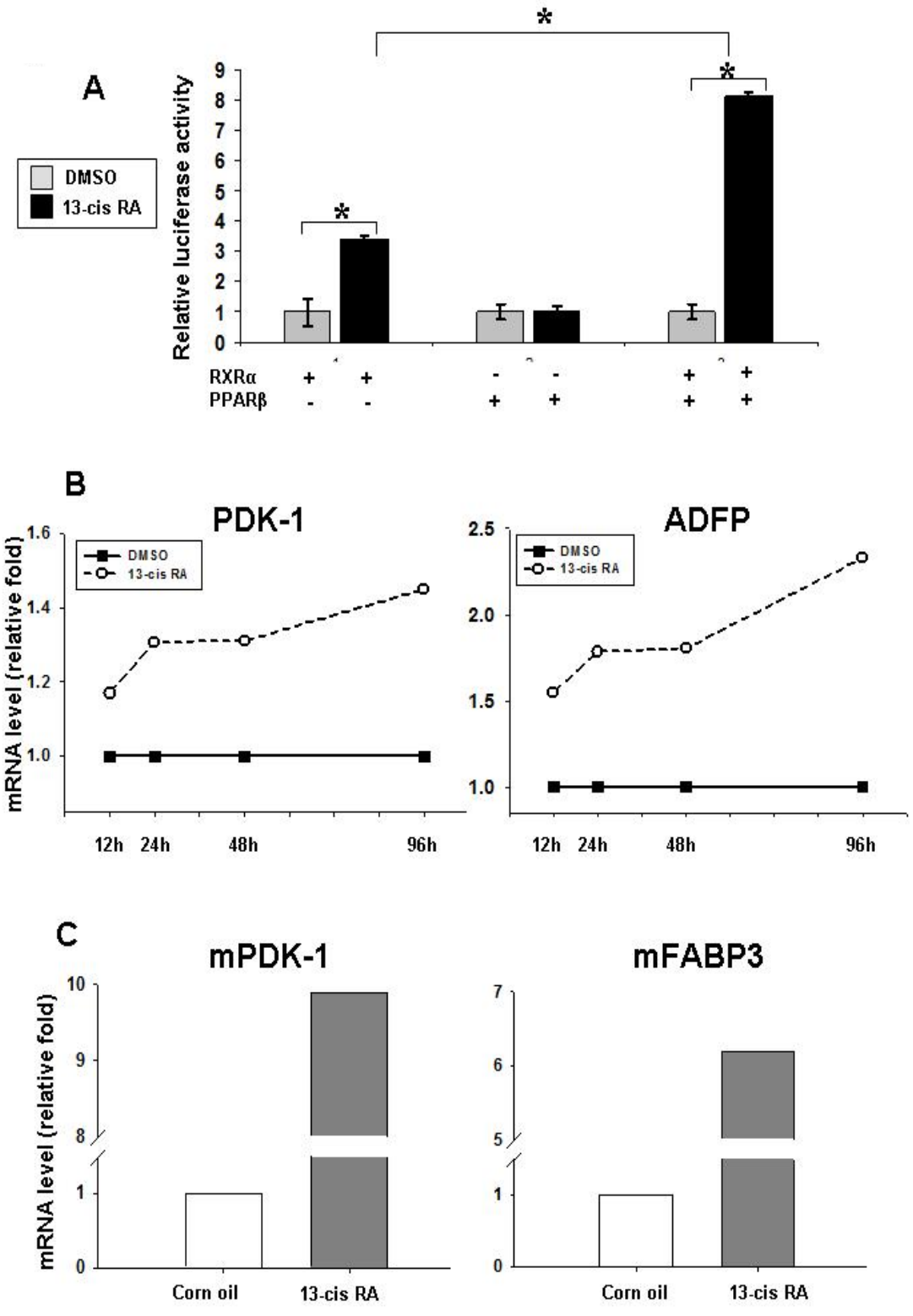
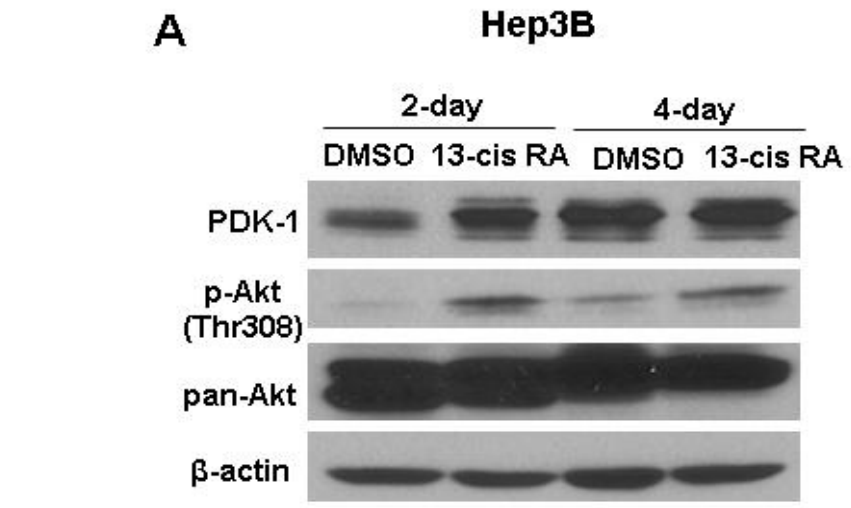

A
DMSO
13-cis RA
Relative luciferase activity
RXRα
PPARβ
B
PDK-1
ADFP
mRNA level (relative fold)
12h
24h
48h
96h
C
mPDK-1
mFABP3
Corn oil
13-cis RA

Figure 5.5: 13-cis RA activated PPARβ as an effective ligand. (A) CV-1 cells were transfected with expression plasmids for RXRα and PPARβ, a firefly luciferase reporter construct harboring PPAR response element (PPRE), and a Renilla luciferase construct. Following transient transfection, cells were treated with or without 13-cis RA for 48hour. Firefly luciferase activity was normalized by Renilla luciferase activity. The histograms depict the fold of normalized firefly luciferase activity relative to DMSO treatment. Data from triplicates were presented as mean ± S.E. PPARβ target genes adipose differentiation-related protein (ADFP) and 3-phosphoinositide dependent protein kinase-1 (PDK-1) and mouse fatty acid binding protein 3 (mFABP3) and mPDK-1 were induced by 13-cis RA in (B) Hep3B cells at the indicated time points and in (C) mouse livers after 10 days, respectively. Gene expression was analysed by quantitative real-time PCR using mRNA from Hep3B cells or livers of mice from the indicated treatments with β-actin as the internal reference. The mRNA level of each gene was expressed as fold relative to DMSO treatment at each time point. Data were presented as mean from duplicates.


A
Hep3B
2-day
4-day
DMSO
13-cis RA
DMSO
13-cis RA
PDK-1
p-Akt
(Thr308)
pan-Akt
β-actin

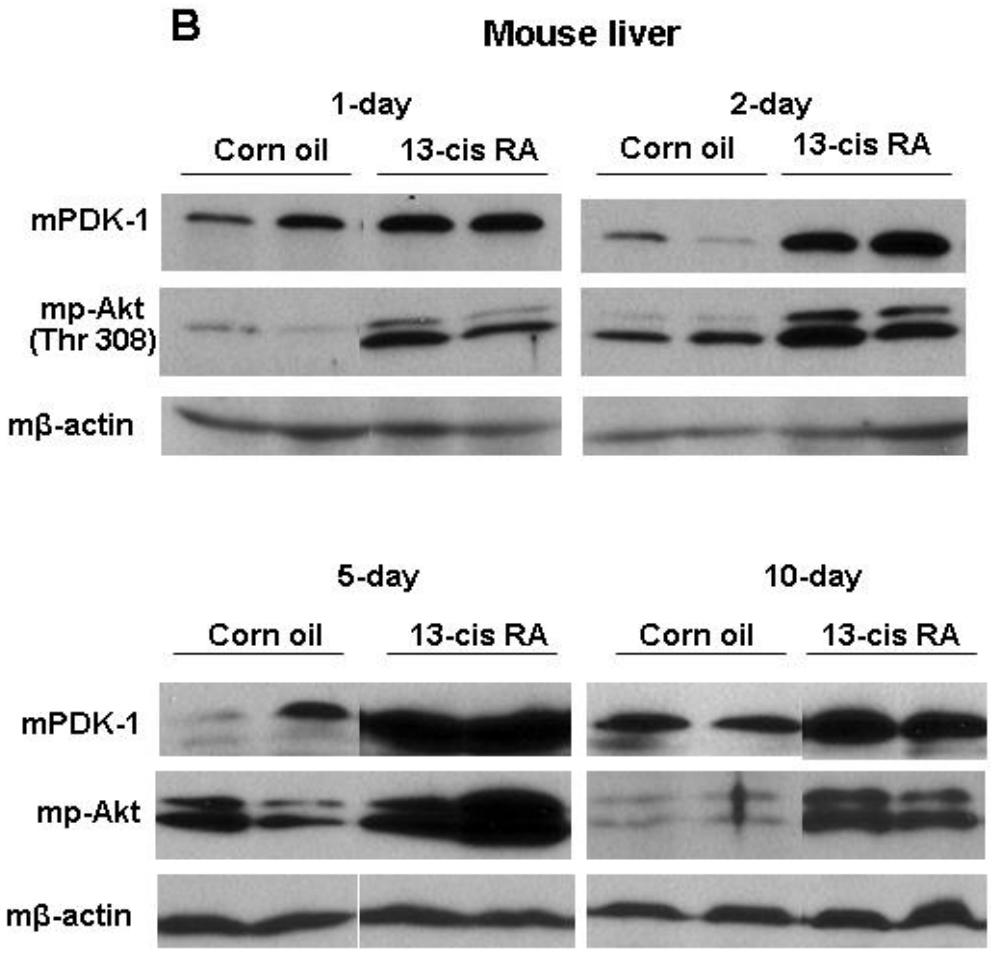
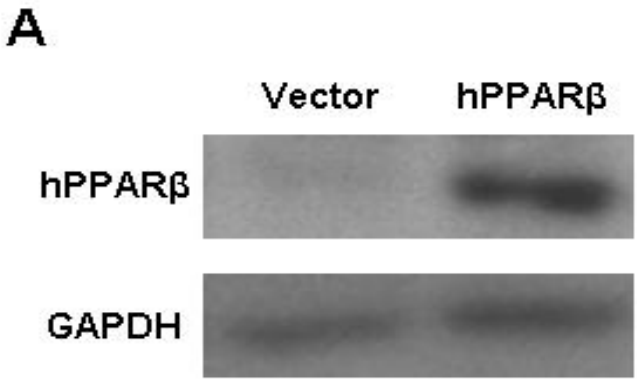

B
Mouse liver
1-day
2-day
Corn oil
13-cis RA
Corn oil
13-cis RA
mPDK-1
mp-Akt
(Thr 308)
mβ-actin
5-day
10-day
Corn oil
13-cis RA
Corn oil
13-cis RA
mPDK-1
mp-Akt
mβ-actin

Figure 5.6: 13-cis RA activated the PPARβ/PDK-1/Akt pathway in both Hep3B cells and mouse livers. Protein levels of PDK-1 and phosphorylated Akt (Thr308) in (A) whole cell extracts of Hep3B cells and (B) total liver protein from mice treated with or without 13-cis RA at indicated time points were analysed by western blotting. Membranes were incubated with antibodies specific to PDK-1 and phosphorylated Akt (Thr308). β-Actin was included as a loading control.


A
Vector
hPPARβ
hPPARβ
GAPDH

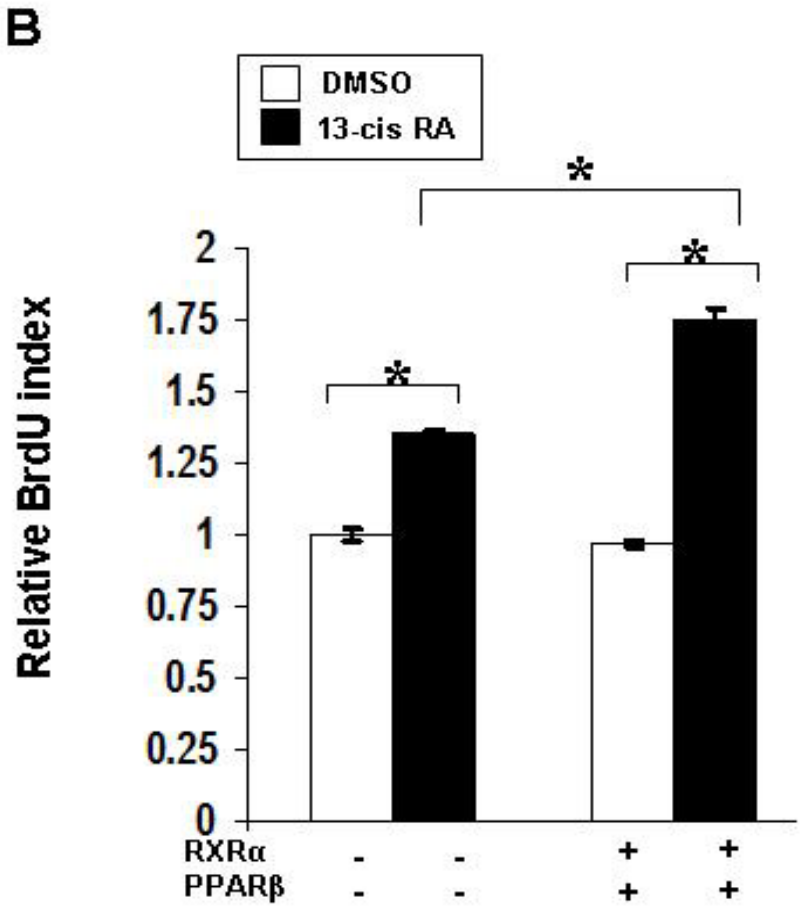
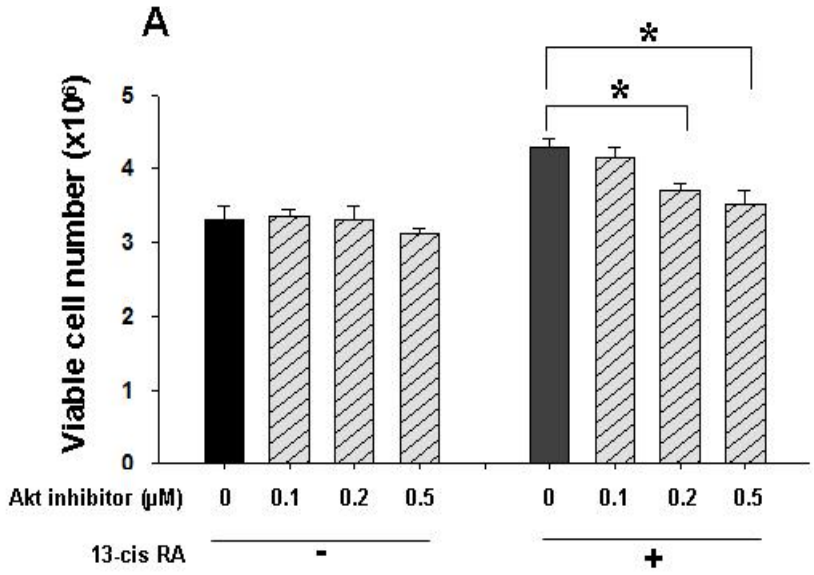
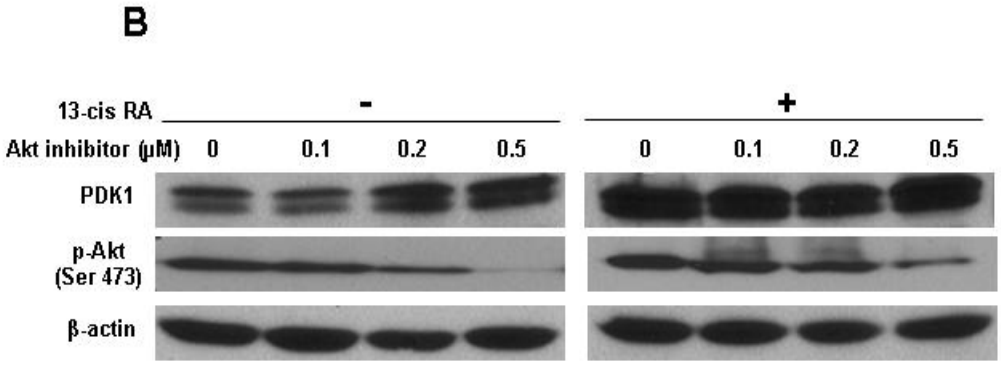

B
DMSO
13-cis RA
Relative BrdU index
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
*
*
*
RXRα - - + +
PPARβ - - + +

Figure 5.7: Overexpression of RXRα and PPARβ further stimulated 13-cis RA-induced proliferation. (A) Overexpression of PPARβ by transfection. Hep3B cells were transfected with either vector or PPARβ expression construct for 48 hours followed by western blotting analysis of whole cell extracts. GAPDH was included as a loading control. (B) Hep3B cells were transfected with indicated constructs for 24 hours and then incubated with or without 13-cis RA for 36 hours followed by BrdU incorporation assay as described in ***Materials and Methods***. Normalized absorbance was expressed as fold ± S.E. relative to vector-transfected DMSO treatment. (* $p <$ 0.05 compared to vector-transfected DMSO treatment). Data from six replicates were presented as mean ± S.E.


A
Viable cell number (x10⁶)
5
4
3
2
1
0
*
*
Akt inhibitor (μM) 0 0.1 0.2 0.5 0 0.1 0.2 0.5
13-cis RA -
+


B
13-cis RA -
+
Akt inhibitor (μM) 0 0.1 0.2 0.5 0 0.1 0.2 0.5
PDK1
p-Akt
(Ser 473)
β-actin

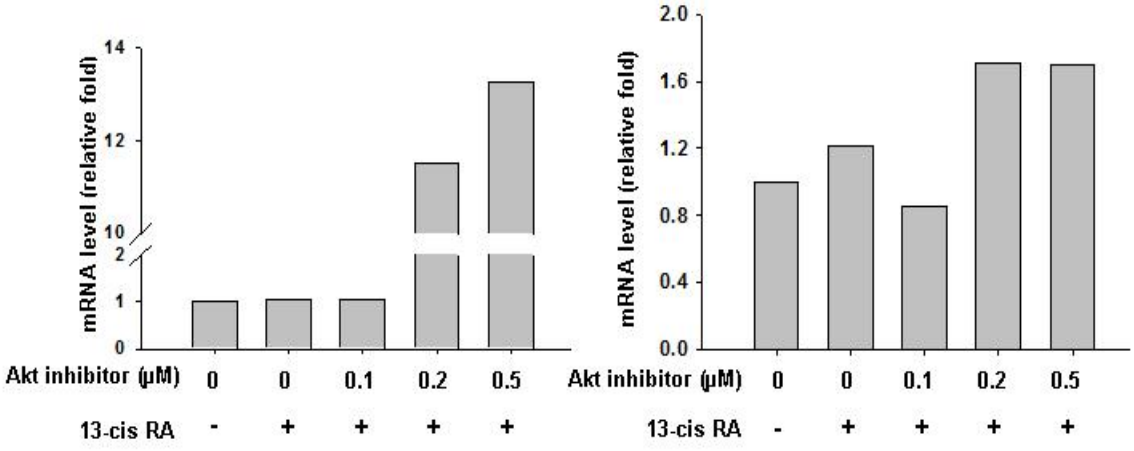

C
mRNA level (relative fold)
14
12
10
2
1
0
Akt inhibitor (μM) 0 0 0.1 0.2 0.5
13-cis RA - + + + +
mRNA level (relative fold)
2.0
1.6
1.2
0.8
0.4
0.0
Akt inhibitor (μM) 0 0 0.1 0.2 0.5
13-cis RA - + + + +

Figure 5.8: Akt inhibitor blocked 13-cis RA-induced Akt activation and proliferation and led to a compensatory induction in the PPARβ/PDK-1/Akt pathway. Hep3B cells were incubated with Akt inhibitor at denoted concentrations with or without 13-cis RA for 72 hours. (A) Viable cell number in the denoted treatments was determined by trypan blue exclusion counting from three replicates and presented as mean ± S.E. (B) Protein level of phospho-Akt (Ser473) in whole cell extracts of Hep3B cells from indicated treatments were analysed by western blotting. Membranes were incubated with an antibody specific to phosphor-Akt (Ser473). β-actin was included as a loading control. (C) Gene expression of PPARβ and PDK-1 was analysed by quantitative real-time PCR with β-actin as the internal reference. The mRNA level of each gene was expressed as fold relative to DMSO alone treatment. Data were presented as mean from duplicates.

Discussion

Reports of the effects of retinoids on hepatocellular carcinoma treatment and prevention and liver regeneration have been conflicting due to use of different retinoids, doses, routes of administration, and animal species (Daoud and Griffin, 1980; Maiorana and Gullino, 1980; Morre et al., 1980; Muto et al., 1996; Tsukamoto, 1999). Several lines of evidence indicate that retinoids stimulate hepatocyte proliferation. (1) Hypervitaminosis A is frequently associated with hepatomegaly (Roenigk, 1989). (2) Administration of all-trans-RA induces hepatocyte proliferation in mice (Ledda-Columbano et al., 2004). (3) All-trans RA, all-trans-ethyl retinamide, and 13-cis-ethyl retinamide significantly increase the incidence of liver tumors (benign and malignant) induced by diethylnitrosamine (Balansky et al., 1994; McCormick et al., 1990). (4) Hepatocyte proliferation is impaired in animals fed a vitamin A deficient diet (Hu et al., 1994). The mitogenic effect of retinoids cautions the their possible usage for anti-tumor purposes, but also implies the usefulness of retinoids in facilitating liver regeneration and liver tissue repair after injury. Thus, it is important to understand the mechanisms underlying retinoid-induced liver cell proliferation.

Given the facts that retinoids are capable of activating different sets of nuclear receptors, it is reasonable to speculate that there are molecular factors within specific cellular context that determine which nuclear receptor sets retinoids is delivered to and which pathway gets preferentially activated. It has been shown that the expression ratio between two cellular binding proteins FABP5 and CRAPB II is

crucial in such a determining process. The low ratio of FABP5/CRABP II in MCF-7 mammary carcinoma cells facilitates the activation of RAR by RA. While in keratinocytes, there is a high ratio of FABP5/CRABP II, which results in the selective delivery of RA to an alternative nuclear receptor PPARβ (Schug et al., 2007). Decreasing the ratio of FABP5/CRABP II in keratinocytes by either overexpression of CRABP II or knockdown of FABP5 converts RA from a survival to a pro-apoptotic factor (Schug et al., 2007). Similarly, in our study, in which human hepatocellular carcinoma cells and *in vivo* mouse livers were examined, the high ratio of FABP5/CRABP II prior to 13-cis RA treatment suggests that PPARβ could mediate the proliferative effect of 13-cis RA. The abundance of FABP5 over CRABP II indicates a possibly preferred activation of PPARβ over RAR by 13-cis RA. Moreover, the ratio of FABP5/CRABP II became even greater after 13-cis RA treatment. However, we cannot rule out the possibilities that other nuclear receptor-mediated pathways including PXR/RXR, RXR/RXR, and PPARα/RXR might also be activated by 13-cis RA. Our previous study along with others' demonstrated that several retinoids can activate human PXR, vitamin D receptor, and PPARα (Keller et al., 1993; Kliewer et al., 1992; Wang et al., 2008; Wang et al., 2006). It is known that the activation of PXR and PPARα is associated with hepatomegaly in rodents (Kliewer et al., 2001; Staudinger et al., 2001; Thatcher and Caldwell, 1994). In addition, it is important to note that the expression of RARβ, a RXR/RARβ target gene and a differentiation marker (Wan et al., 1995), was also induced by 13-cis RA in Hep3B cells and mouse livers (data not shown). This finding indicates that in

hepatocellular carcinoma cells and mouse livers, 13-cis RA also activates RARβ-mediated pathway to some extent. If the activation of RARβ and PPARβ has opposite effects, it seems that in adult liver, which is well differentiated and fully matured, the proliferative effect of PPARβ can override the differentiation effect of RARβ in response to 13-cis RA. It is also important to note that other retinoic acids such as 9-cis RA and all-trans RA do not exert a proliferative effect in Hep3B cells (Bu and Wan, 2007; Wan et al., 1998). However, both 9-cis RA and all-trans-RA are mild mitogens in rodent livers (Ledda-Columbano et al., 2004; Ohmura et al., 1996). Thus, it seems the mitogenic effect of RA is stronger *in vivo* than *in vitro*, part of the answer may lie in the differentiation and maturation status of the hepatocytes. In addition, the proliferative effect of retinoids appears to be isomer-specific.

Previous studies have demonstrated that all-trans RA is a highly selective ligand for PPARβ with nanomolar affinity in transactivation assay in COS-7 cells (Shaw et al., 2003). Upon binding, all-trans RA modulates the conformation of PPARβ, promotes its interaction with the coactivator SRC-1, and efficiently activates PPARβ-mediated transcription (Shaw et al., 2003). Our data demonstrated that besides all-trans RA, another natural retinoid, 13-cis RA, is also able to activate PPARβ in hepatocellular carcinoma cells and in liver.

Despite some discrepancies in the literature, PPARβ appears to possess proliferative effect in keratinocytes, breast cancer and prostate cancer cells (Di-Poi et al., 2003; Di-Poi et al., 2002; Icre et al., 2006; Stephen et al., 2004). Our data demonstrates for the first time that PPARβ also has a proliferative effect in liver cells.

The survival-promoting effect of PPARβ is mediated in part by direct transcriptional induction of its target gene, PDK-1, and downstream activation of Akt (Di-Poi et al., 2002; Tan et al., 2004). PDK-1 phosphorylates Akt at Thr308 in its activation loop, which serves as a direct "on/off" switch (Toker and Newton, 2000). Thus, it appears that the nuclear receptor PPARβ and its downstream mediators transduce a potent survival/proliferation signal. Similarly, we demonstrated 13-cisRA-induced PPARβ activation further leads to PDK-1 induction and Akt activation. Results from over-expression of RXRα/PPARβ and pharmacological intervention of Akt further confirmed that the proliferative effect of 13-cis RA is mediated through PPARβ/PDK-1/Akt pathway.

The activation of PPARβ/PDK-1/Akt pathway has profound biological consequences in epithelial carcinogenesis (Burdick et al., 2006). PPARβ expression is negatively regulated by the tumor suppressor gene adenoma polyposis colitis (APC) in colorectal tumor cells (He et al., 1999). Administration of PPARβ ligands suppresses apoptosis in colon cancer cells and enhances small intestine tumorigenesis in the APC^{min} mouse model (Gupta et al., 2004; Wang et al., 2004). In addition, an essential role for PPARβ has also been established in tumor stroma and tumorigenesis. PPARβ is required for functional tumor blood vessel formation by regulating tumor endothelial cell proliferation and differentiation (Muller-Brusselbach et al., 2007; Muller et al., 2008). PPARβ null mice display an impaired growth of transplanted Lewis lung carcinoma compared to wild type mice. The concomitant deregulated tumor angiogenesis in PPARβ null mice appears to result from diminished blood flow

and abundant endothelial hyperplasia (Muller-Brusselbach et al., 2007).

About 50-80% of total body retinoids (retinol and retinyl esters) are stored in the liver stellate cells (Blomhoff and Blomhoff, 2006). During hepatic injury, stellate cells are activated and the transdifferentiation of stellate cells into fibroblast is associated with vitamin A depletion (Bataller and Brenner, 2005; Mabuchi et al., 2004). The released vitamin A should be converted into its functional active form, i.e. retinoic acid, by retinoid metabolizing enzymes. Whether retinoic acid can activate PPARβ pathway and play a role in hepatocyte proliferation during liver regeneration warrants further investigation.

CHAPTER SIX

GENERAL DISCUSSION

OUTLINE

6.1 Termination of retinoid signaling by the ubiquitin-proteasome system and future directions

6.2 Mechanism of fenretinide-induced apoptosis of HCC cells

6.2.1 Phosphorylation of RXRα and activation of mitogen-activated protein kinases (MAPK)

6.2.2 Potential interaction between RARβ and Nur77 and future directions

6.3 Retinoid-induced hepatocyte proliferation

6.3.1 Dual roles of Akt in retinoid signaling

6.3.2 Roles of PXR, CAR, and PPARα in retinoid-induced hepatocyte proliferation

6.1 Termination of retinoid signaling by the Ubiquitin-proteasome system and future directions

In the past two decades, extensive investigations have focused on how nuclear receptor-mediated retinoid signaling is initiated and amplified but relative little attention has been devoted to the mechanisms of signal termination.

It has become evident that the transcriptional activity of retinoid receptors, as that of most transcription factors, is regulated by the ubiquitin–proteasome pathway (Bastien and Rochette-Egly, 2004). One main role of the ubiquitin–proteasome system is to degrade proteins. In general, following some signals, the substrate protein is multi-ubiquitylated at lysine groups and then targeted for destruction by the 26S proteasome. The 19S sub-complex of the proteasome recognizes the multi-ubiquitylated substrate, removes the ubiquitin groups, unfolds the substrate protein and feeds the resulting unstructured chain into the 20S catalytic core of the proteasome where it is degraded (DeMartino and Slaughter, 1999). It is reasonable to speculate that an important role of the ubiquitin-proteasome is to degrade ligand-bound nuclear receptors and thereby terminate the ongoing signal transduction. However, paradoxical roles for the ubiquitin–proteasome system in the regulation of RARs actions were reported. Blocking either the ubiquitin or the proteasome systems abrogated not only the degradation of RARγ, but also RARγ-mediated transcription. This is surprising as it would be anticipated that enhanced RARγ-mediated transcription would occur when RARγ accumulates within the cell due to blockade of its degradation. Therefore, in this case, the ubiquitin–proteasome machinery seems to

play a dual role, controlling on the one hand the functionality of RXR/RARγ heterodimers, and on the other hand the degradation of the heterodimers. It is worth mentioning that the same conclusions could not be made for RARα. Inconsistently, inhibition of the proteasome by specific inhibitors did not abrogate, but rather amplified RARα-mediated transcription (Bastien and Rochette-Egly, 2004). Therefore, further investigations are required regarding why RARα and RARγ are not regulated similarly by the ubiquitin-proteasome system.

A more relevant question to our study is how fenretinide signaling is terminated in HCC cells. In order to examine the role of the ubiquitin-proteasome system in degrading RARβ and/or Nur77 during fenretinide-induced apoptosis of HCC cells, we can employ the pharmacological inhibitors for the ubiquitin-proteasome system and assess the corresponding outcomes. For example, if fenretinide-induced apoptosis and the cellular levels of RARβ and/or Nur77 are enhanced in the presence of the inhibitor, it suggests that the ubiquitin-proteasome system plays a role in the termination of fenretinide signaling by degrading the fenretinide-activated RARβ and/or Nur77. However, it is also possible that the presence of the inhibitor may cause a reduced phenotype, which indicates a positive role of ubiquitin-proteasome system such as eliminating certain inhibitory factors in fenretinide-induced apoptosis of HCC cells. The proposed experiments will provide mechanistic insights into the regulation of the termination of fenretinide signaling studied in this dissertation.

6.2 Mechanism of fenretinide-induced apoptosis of HCC cells

6.2.1 Phosphorylation of RXRα and activation of MAP Kinases

It has been established that RARs and RXRs are substrates for several protein kinases (Rochette-Egly, 2003). RARα and RARγ are phosphorylated by the cdk7 subunit of the general transcription machinery during transcription initiation. Inhibition of phosphorylation leads to hypophosphorylation of RARα and reduced transduction of retinoid signaling (Bastien and Rochette-Egly, 2004). The phosphorylation of nuclear receptors during the assembly of the transcription complex is generally believed to have two functions: (1) phosphorylation could facilitate the recruitment of components of the transcription machinery and further stabilize the formation of the nuclear receptor-transcription complex; and (2) phosphorylation might rather facilitate the dissociation of nuclear receptors from transcription corepressors or help the dissociation of nuclear receptors from the transcription machinery in order to allow elongation to proceed (Bastien and Rochette-Egly, 2004).

Phosphorylation of nuclear receptors in the above scenarios is dynamic and tightly regulated. However, a distinct pattern of phosphorylation of RXRα has been discovered. Studies have revealed that phosphorylation of serine 260 residue of RXRα, which locates in the Omega loop of RXRα between helices H1 and H3 and close to helix 12, impairs the transcriptional activity of RXR/RAR heterodimer and thereby causes resistance of cancer cells to retinoid signaling-induced growth

inhibition (Lee et al., 2000; Matsushima-Nishiwaki et al., 2001; Matsushima-Nishiwaki et al., 2003; Solomon et al., 1999). In addition, in HCC cells, phosphorylation of RXRα at Serine 260 results in its resistance to proteolytic degradation and abrogation of this phosphorylation event by MAP kinase (ERK1/2 and p38) specific inhibitors restored the degradation of RXRα in an RXR ligand-dependent manner (Matsushima-Nishiwaki et al., 2001). Therefore, the phosphorylation of RXRα at Serine 260 interferes with its metabolism as well as the retinoid signaling that it mediates in HCC cells. Similarly, we have found that the constitutive phosphorylated RXRα (Serine 260) in Huh-7 HCC cells was reduced in response to fenretinide treatment. Furthermore, the reduction in phosphorylated RXRα was only detected in the sensitive cells with no change occurring in the resistant HepG2 cells before and after fenretinide treatment. Therefore, in the HCC cells that we have tested, the level of RXRα phosphorylation correlates well with the susceptibilities of HCC cells to fenretinide-induced apoptosis. This may provide an explanation for the observed phenotypes. When phosphorylation of RXRα (Serine 260) is reduced, partial restoration of the endogenous retinoid signaling occurs which may render these cells become sensitive to fenretinide. But this conclusion is only valid under the condition where RXRα is the primary mediator of fenretinide effect in HCC cells and the reduction of RXRα phosphorylation occurs relatively early and upstream of the initiation of apoptosis.

RXRα has been demonstrated as a substrate for ERK and p38 and Serine 260 is a consensus site for MAP kinases (Solomon et al., 1999). Furthermore, inhibition of

MAP kinase activity with pharmacological inhibitors or point mutagenesis of serine 260 in RXRα abolishes MAP kinase-mediated RXRα phosphorylation. Therefore, these findings reveal another layer of RXRα phosphorylation regulation, i.e. at the level of MAP kinases. We have found that the activation of MAP kinases also correlates with the differential susceptibilities of HCC cells to fenretinide treatment. In Huh-7 cells, fenretinide reduced phosphorylated/activated ERK in a time-dependent manner and thus dampened ERK-mediated survival pathway in the sensitive cells. Whereas the same retinoid continuously increased ERK activation in HepG2 cells and thus enhanced ERK-mediated survival pathway in the resistant cells. Taken together, the patterns of both MAP kinase activation and RXRα phosphorylation correlate with the observed susceptibilities of HCC cells to fenretinide, and thus, suggest a protective/anti-apoptotic role of ERK in fenretinide-induced apoptosis. However, a recent study revealed that the activation of ERK in response to fenretinide is required for apoptosis induction in head and neck cancer cells, because inhibition of ERK with a specific inhibitor blocked the apoptosis induced by fenretinide (Kim et al., 2006). This apparent discrepancy may be partially due to different cell types, as it was clearly demonstrated that fenretinide generated reactive oxygen species (ROS) in head and neck cancer cells shortly after treatment. Generation of ROS further triggers the activation of MAP kinases in these cells. However, ROS generation was relatively mild in HCC cells in response to fenretinide. Some preliminary data generated in our lab demonstrated the combination of fenretinide and ERK inhibitor further elevated ROS generation and enhanced

apoptosis induction. Therefore, these findings suggest a protective/anti-apoptotic role of ERK and a pro-apoptotic role of ROS in fenretinide-induced apoptosis. One of the future experiments will be to investigate the downstream effectors of ROS. The stress-activated kinase p38 may serve as a promising candidate. In the presence of fenretinide alone and fenretinide plus ERK inhibitor, if the phosphorylation/activation of p38 positively correlates with the enhanced apoptosis induction, it strongly suggests the involvement of p38 in this process.

6.2.2 Potential interaction between RARβ and Nur77 and future directions

The interaction between nuclear receptors RARβ and Nur77 has not yet been extensively explored in the literature. One study showed that Nur77 inhibited RARβ induction by antagonizing COUP-TF (Wu et al., 1997). Our findings suggest that both nuclear receptors are involved in mediating fenretinide-induced apoptosis in HCC cells. The comparison of time courses of RARβ and Nur77 mRNA induction reveals that RARβ was induced 2 hours after the treatment and its induction was sustained time-dependently and reached 35-fold at the end of 48-hour treatment. On the other hand, Nur77 was mildly induced after 6 hours of fenretinide treatment, and its overall induction was less than that of RARβ (7-fold after 48 hours). Therefore, it is possible that Nur77 may be downstream of RARβ, as its induction was slower and weaker than that of RARβ. Another possibility is that Nur77 works upstream of RARβ. This possibility is supported by the reported evidence that Nur77 forms heterodimers with RXRα and recognizes RARE (DR5) as the cognate DNA response

element (Maxwell and Muscat, 2006). It is also known that the RARβ promoter contains a functional RARE (DR5). In order to determine the transcriptional connection between these two nuclear receptors, one approach would be to knock down the endogenous RARβ and Nur77 by siRNA and monitoring the expression of Nur77 and RARβ before and after fenretinide treatment. The third possibility comes from a transcription-independent perspective. It is possible that there is not much connection between RARβ and Nur77 at the transcriptional level, but instead, RARβ regulates mitochondrial targeting of Nur77 during fenretinide treatment. This hypothesis can be examined by monitoring Nur77 intracellular localization in RARβ deficient cells following fenretinide treatment. Either immunofluorescence staining for Nur77 or subcellular fraction isolation will provide direct evidence to prove or disprove this hypothesis.

6.3 Retinoid-induced hepatocyte proliferation

6.3.1 Dual roles of Akt

Our results demonstrate an essential role of Akt along the PPARβ/δ pathway in mediating 13-cis retinoic acid-induced hepatocyte proliferation (Chapter 5). The inhibition of Akt with a pharmacological inhibitor effectively blocked 13-cis retinoic acid-induced proliferation of Hep3B cells. However, another study reported an differentiation-associated role for Akt in retinoic acid-induced differentiation in mouse embryonic carcinoma cells (F9 cells) (Bastien et al., 2006). These authors observed an early activation of phosphoinositide 3-kinase (PI3K) and its downstream Akt in response to retinoic acid. But this effect is followed by an inhibition of Akt. Furthermore, both the activation and inhibition of Akt require a functional retinoic acid signaling pathway. A model of Akt action was proposed in F9 cells where retinoic acid induced a biphasic regulation of Akt: with the activation of Akt participating in the differentiation process and the inhibition of Akt contributing to retinoic acid-induced growth inhibition (Bastien et al., 2006). The PI3K/Akt pathway has important roles in various cellular processes such as proliferation and cellular survival (Brazil et al., 2004). Whether this observed differentiation-associated role of Akt in retinoic acid-induced differentiation is universal or only specific to F9 cells needs to be further investigated.

6.3.2 Roles of PXR, CAR, and PPARα in retinoid-induced hepatocyte proliferation

Xenobiotics produce many effects via activation of CAR (constitutive androstane receptor) and PXR (pregnane X receptor), the so-called "xenobiotic sensors". The human ortholog of PXR is SXR (steroid and xenobiotic receptor). Mouse PXR ligand pregnenolone 16α-carbonitrile (PCN) induces hepatomegaly in wild type mice, but not in PXR null mice (Staudinger et al., 2001). Constitutive activation of human PXR/SXR in PXR null mice also results in hepatomegaly (Xie et al., 2000). Similarly, the hepatomegaly effect of 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), the ligand for rodent CAR, is completely abolished in CAR knockout mice (Wei et al., 2000). In rodents, peroxisome proliferator-activated receptor α (PPARα) agonists cause peroxisome proliferation and hepatomegaly (Kliewer et al., 2001). We examined the activation of nuclear receptors SXR, CAR, and PPARα in Hep3B cells and mouse livers following 13-cis retinoic acid treatment. Some of those nuclear receptor target genes were induced. We cannot rule out the possibilities that those nuclear receptor-mediated pathways might also contribute to the proliferative effect of 13-cis retinoic acid. It has been demonstrated that liver injury induced by either chemical or surgical removal will result in hepatocyte proliferation to compensate for the loss of liver mass and function. For example, in mice, after two-thirds partial hepatectomy (PH), the remnant liver is able to initiate robust proliferation and grows back to its original size within 7-10 days after surgery (Black et al., 2004; Fausto and Riehle, 2005; Michalopoulos and

DeFrances, 1997; Zimmermann, 2004). In our studies, mice were given retinoid by gastric gavage once a day for 5 and 10 days. The hepatic stress is significantly less compared to the two-thirds PH. Therefore, there is no urgent physiological or necessary pathological need for the liver to regenerate as it does in the two-thirds partial hepatectomy paradigm. As expected, the hepatocyte proliferation triggered by retinoid in our *in vivo* model is much milder when compared to the massive liver regeneration induced by two-thirds PH. However, the current study demonstrated for the first time the hepatic mitogenic effect of 13-cis RA, which is clinically used to treat skin disorders such as psoriasis. Our findings raise a concern for long term usage of 13-cis retinoic acid and also provide an explanation for the long-observed vitamin A intoxication-induced hepatomegaly.

Concluding Statements

Retinoids, in addition to being essential nutrients for human health, are also important signaling molecules participating in regulation of homeostasis at cellular and tissue levels. Given the broad chemical and physiological diversities of retinoids and the numerous pathways activated by them, retinoid biology posses a vast and complicated research field to explore. Thus, data from multiple experimental systems will provide valuable insights into both general and specific mechanisms underlying retinoid actions. In this dissertation, we examined the versatile effects of retinoids in liver cells and investigated the underlying mechanisms responsible for this diversity. The overall finding of this dissertation is that retinoids can cause opposing effects in liver cells. These effects are retinoid-specific, mediated by distinct nuclear receptors, as well as intrinsic cellular context-dependent. Fenretinide, a synthetic retinoid, has been evaluated in several clinical trials but its role in treating liver malignancy has not been extensively investigated. The results from Chapter 3 and 4 provide a strong rationale for the clinical application of fenretinide in liver cancer. Furthermore, the findings establish a role for the nuclear receptors RARβ and Nur77 in mediating fenretinide-induced apoptosis. Deregulation of both nuclear receptors was observed in cells resistant to fenretinide-induced apoptosis. Therefore, the first part of the dissertation demonstrates that fenretinide could be a useful chemotherapeutic agent for treating liver cancer and the levels of RARβ and Nur77 may be used as biomarkers to predict and monitor the responsiveness of the patients to fenretinide treatment. The second part of the dissertation investigated the mechanism responsible

for retinoid-induced hepatocyte proliferation. The results from Chapter 5 demonstrate that the activation of a signaling cascade PPARβ/PDK-1/Akt is responsible for 13-cis retinoic acid-induced proliferation of liver cells. Thus, the present findings establish a mechanism that is responsible for the long-observed retinoid-induced hepatomegaly, which further raises some caution regarding the clinic use of 13-cis retinoic acid.

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